Homeobox D10 Induces Phenotypic Reversion of Breast Tumor Cells in a Three-Dimensional Culture Model

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Abstract
Homeobox (Hox) genes are master regulatory genes that direct organogenesis and maintain differentiated tissue function. We previously reported that HoxD10 helps to maintain a quiescent, differentiated phenotype in endothelial cells by suppressing expression of genes involved in remodeling the extracellular matrix and cell migration. Here we investigated whether HoxD10 could also promote or maintain a differentiated phenotype in epithelial cells. We observed that HoxD10 expression is progressively reduced in epithelial cells as malignancy increases in both breast and endometrial tumors. Retroviral gene transfer to restore expression of HoxD10 in the malignant breast tumor cells MDA-MB-231 significantly impaired migration, and when these cells were cultured in a three-dimensional laminin-rich basement membrane (3DlrBM) model, they formed polarized, acinar structures. This phenotypic reversion was accompanied by decreased α3 integrin expression and reduced proliferation. Importantly, expression of HoxD10 in the MDA-MB-231 cells inhibited their ability to form tumors in mouse xenografts. Taken together, our results suggest that HoxD10 has tumor-suppressive functions for mammary epithelial cells. (Cancer Res 2005; 65(16): 7177-85)

Introduction
Type I class Homeobox (Hox) genes encode master transcription factors that play an important role in organogenesis and morphogenesis during development (1). In addition to their role in embryogenesis, Hox genes are expressed in adult cells where they regulate expression of genes involved in cell proliferation as well as cell-cell and cell-extracellular matrix interactions (2–9). It is now well established that several Hox genes display an altered pattern of expression in certain malignancies as compared with normal tissue including leukemia and solid tumors such as breast, endometrium, brain, colon, prostate, lung, and kidney (10–15). Although it was originally believed that up-regulation of Hox genes promoted oncogenesis, it is now becoming evident that loss of various Hox genes is also associated with tumorigenesis (16, 17). Loss of normal breast tissue architecture and polarity, a pathologic hallmark of breast cancer, is due to altered interactions between cancer cells and their surrounding extracellular matrix (18). In the normal mammary gland, Hox genes play an important role in dictating cell specificity, proliferation, and differentiation during mamopoiesis, both in development and in pregnancy (19). Thus, considering the role for Hox genes in regulating breast tissue morphogenesis and cell-extracellular matrix interactions in normal tissues, it is likely that loss of Hox gene expression might underlie changes in cell-extracellular matrix interactions, which give rise to the aberrant morphology and behavior of breast tumors. Indeed, there are numerous examples of aberrant expression of Hox genes in mammary tumors. Whereas HoxC6 is present in normal breast tissue, it is absent in mammary neoplasia (7, 13). HoxA5 expression is also lost in breast cancer. Raman et al. (17) reported that methylation of the HoxA5 promoter correlates with loss of p53 expression in breast tumors, and Cheng et al. (20) showed that reexpression of HoxA5 can induce apoptosis through an apoptotic mechanism mediated by caspases 2 and 8. On the other hand, HoxA1 is detected in mammary tumors, but not in the normal gland or in precancerous outgrowths (7, 13, 21). It has been shown that forced expression of HoxA1 in human mammary carcinoma cells results in oncogenic transformation with in vivo tumor formation (22). To date, however, no studies have shown that restoring normal Hox gene expression can influence the course of breast tumorigenesis in vivo.

Work from our laboratory showed that sustained expression of HoxD10 suppresses angiogenesis in vivo, suggesting that HoxD10 contributes to maintenance of a quiescent, differentiated phenotype in endothelial cells (3). Specifically, HoxD10 suppresses expression of genes that directly affect remodeling of the extracellular matrix and cell migration during angiogenesis such as α3 integrin, matrix metalloproteinase 14 (MMP-14), and urokinase-type plasminogen activator receptor (uPAR). Interestingly, these genes have been shown to promote invasion, migration, and tumor progression in a variety of cells, including breast tumors (23–27), raising the possibility that HoxD10 functions as a general inhibitor of cell invasion.

In this study, we investigated whether HoxD10 expression is reduced in tumorigenic epithelial cells and whether restoring its expression can redirect normal tissue morphology and help maintain a quiescent differentiated state in epithelial cells. We show that HoxD10 is lost during tumor progression both in breast and endometrial tumors. In addition, HoxD10 is capable of reverting the highly invasive breast tumor cell line MDA-MB-231 to a nonmalignant state by restoring normal cell-cell and cell-extracellular matrix interactions when culturing the cells in the three-dimensional laminin-rich basement membrane (3DlrBM) assay. Moreover, HoxD10 suppresses tumor growth in vivo in a mouse xenograft model.

Materials and Methods
Cell culture. The human breast epithelial cell line MDA-MB-231 and the human cervical epithelial (HeLa) cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Both cell lines were maintained in DMEM/F12 (Life Technologies, Rockville, MD) with 5% fetal bovine serum and 5 μg/ml gentamicin sulfate (Life Technologies). Three-dimensional cultures were prepared using a modified procedure described by Weaver et al. (28). Briefly, cells were grown to confluence as monolayers, trypsinized, and plated (3 × 10^4 cells in 24-well plates) on top
of a 300 μL of polymerized Matrigel (10.75 mg/mL; BD Biosciences, Bedford, MA) in 0.5 mL DMEM/F12 media without serum and with 250 ng/mL insulin (Sigma, St. Louis, MO). After 30 minutes of incubation at 37°C, 0.5 mL media containing 10% Matrigel was added on top of the cells. Cultures were analyzed after 4 days of cultivation. The morphology of the three-dimensional cultures was assessed by visual examination of the degree of colony organization with phase-contrast microscopy.

**Retroviral vectors and transduction.** The human L100 bp HoxD10 cDNA (GenBank accession no. X59373) or a HoxD10 DNA binding mutant which contains a N316T [asparagine (N)-316 to threonine (T)] point mutation in the second helix of the homeodomain, abolishing DNA binding (SwissProt accession no. P28358, a gift from Dr. C. Largman, University of California San Francisco, San Francisco, CA), was cloned into the EcoRI site of the pLXSN retroviral vector (Clontech, Palo Alto, CA). The resultant vectors were confirmed by DNA sequencing at the Biomolecular Resource Center, University of California San Francisco, and designated pLD10 and pLD10 N-T, respectively. Retroviral vectors were transfected by the calcium/phosphate-DNA precipitation method into the amphotropic packaging cell line Phoenix Amphi (ATCC), and 48 hours after transfection, viral supernatant was collected, passed through 0.45 μm filters, and used for transduction.

**Release of cellular structures and RNA extraction from three-dimensional cultures.** Cultures grown in three-dimensions were released from Matrigel using a modified procedure described by Weaver et al. (28). Briefly, cells were washed with cold PBS without Ca2+ and Mg2+ and containing 5 mmol/L EDTA, and scraped into a centrifuge tube with a minimum volume of 30 mL cold PBS/EDTA. Cells were incubated on ice for 45 minutes, until the Matrigel disassociated, and centrifuged at 115 × g for 2 minutes. The pellet was resuspended in an appropriate amount of RNA lysis buffer and RNA extraction was done using the RNeasy isolation kit (Qiagen, Valencia, CA).

**Reverse transcription-PCR.** One microgram of total RNA extracted by RNeasy isolation kit (Qiagen) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Qiagen), and 1/25 of this reaction was linearly amplified for 30 cycles (HoxD10 and IF integrin), 25 cycles (α3 integrin and uPAR), and 32 cycles (MMP-14) following denaturation (30 seconds at 95°C), annealing (30 seconds at 56°C for HoxD10, IF integrin, and α3 integrin; 60°C for MMP-14 and uPAR), and extension (30 seconds at 72°C) in a thermal cycler (PTC-200 Peltier Thermal cycler, MJ, Research, Waltham, MA). The following primers were used: HoxD10 forward 5′ CTGTCATGCTCCAGCAACC3′, reverse 5′ CTGAAAGAACGTTGGTGGCCTC3′; IF integrin forward 5′ATGGACTTCTTCCACACTCC3′, reverse 5′ GAGGATTGGTGCTGAAAGAAG3′; α3 integrin forward 5′ CAAGTGTCCTGATCCACACC3′, reverse 5′ CTGTCCTTCCACACTCC3′; IF integrin forward 5′ ATGGACTTCTTCCACACTCC3′, reverse 5′ GAGGATTGGTGCTGAAAGAAG3′; α3 integrin forward 5′ CAAGTGTCCTGATCCACACC3′, reverse 5′ CTGTCCTTCCACACTCC3′; MMP-14 forward 5′ CAACACTGCTCAAGGAGGA3′, reverse 5′ GTTCTA CCTTACGCTTCTG3′ and uPAR forward 5′ CCTTACGCTTCTGTAACACTGG3′, reverse 5′ CGAGCACTTGTGCAGGACAC3′. Total RNA was normalized using 18S internal standards at a 1:4 ratio (Ambion, Austin, TX).

**Immunoblot analysis.** Cells were grown in 3D BM cultures for 4 days, and colonies were isolated using ice-cold PBS/EDTA as mentioned before. Pellets were formed by lysate in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L sodium chloride, 1% NP40, 0.5% deoxycholate, 0.2% SDS, 20 mmol/L sodium fluoride, and 1 mmol/L sodium orthovanadate, and a cocktail of protease inhibitors]. Equal amounts of protein were separated on a 7.5% nonreducing SDS-PAGE gel, immuno-blotted with a polyclonal α3 integrin antibody (AB1920, Chemicon, Temecula, CA) or polyclonal IF integrin antibody (AB1922, Chemicon), and detected with an enhanced chemiluminescence system (Amersham Pharmacia BioTech, Piscataway, NJ).

**Analysis of proliferation.** Cells (5 × 104) were plated in 12-well plates and serum starved for 16 hours. Proliferation was evaluated by adding 1 mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)/media (15% MIT/BSA 2 mg/mL and 85% media; Sigma) into each well. After 3 hours of incubation at 37°C in the dark, MTT was removed and 500 μL of solubilization solution (0.04 N HCl in isopropanol and 3% w/v SDS) were added. After 1 hour at 37°C in the dark, 150 μL of each sample were transferred into 96-well plates and the absorbance was determined at 600 nm using a Wallac Victor2 1420 Multilabel Counter (Perkin-Elmer Life Sciences, Boston, MA). In addition, we also did analysis of BrdUud incorporation as previously described (29).

**Transwell migration assay.** Cell migration assays were done in modified Boyden chambers. Briefly, 6.5 mm transwell chambers with 8 μm pores (Corning, Acton, MA) were coated with 10 μg/mL of type I collagen (Collagen Biomaterials, Palo Alto, CA) for 2 hours at 37°C. Cells were serum starved for 16 hours and 5 × 103 cells/well were plated to the upper chamber in 300 μL of fibroblast basal medium (Clonetics, San Diego, CA) containing 0.5% bovine serum albumin (BSA). The lower chamber was filled with 500 μL of fibroblast basal medium. After 4 hours at 37°C, nonmigratory cells in the upper chamber were removed with a cotton swab and cells that migrated to the bottom of the membrane were fixed and stained with Diff-Quick (VWR Scientific Products, West Chester, PA). The total number of migrated cells was determined by counting five fields in each well per experimental condition using a phase-contrast microscope (magnification, ×20).

When indicated, cells were preincubated for 2 hours at 37°C with 5 μg/mL of control immunoglobulin G (IgG) or function-blocking antibodies against mouse anti-human integrin α3 (Chemicon) or monoclonal antibody (mAb) against human uPAR (Clon CD87, American Diagnostica, Inc., Greenwich, CT). After preincubication, cells were plated to the upper chamber and migration analysis was done as described earlier. When indicated, 10 μg/mL of GM6001 (Ihomastat) MMP inhibitor (Chemicon) was added to the cells just before plating them into the transwell.

**Immunofluorescence.** Three-dimensional cultures were fixed in 2% paraformaldehyde at room temperature for 1 hour, embedded in 20% sucrose, and frozen in Tissue-Tek optimum cutting temperature compound (Sakura Finetek USA, Inc., Torrance, CA). Five-micrometer-thick frozen sections were prepared for immunostaining as follows: sections were fixed with acetone for 30 seconds at room temperature, washed with 50 mmol/L PBS/glycine thrice for 20 minutes at room temperature, blocked with 10% goat serum in immunofluorescence buffer (0.1% BSA, 0.2% Triton X-100, 0.05% Tween 20 in PBS) for 1 hour at room temperature, and with 2.5% BSA with 1:100 fab fragment [anti-mouse IgG, F(ab’)2 fragment of goat antibody, Sigma] in immunofluorescence buffer for 45 minutes at room temperature. Incubation with the primary antibody was done at 1:100 dilution of antibody in immunofluorescence buffer plus 1:100 fab fragment for 1 hour at room temperature. Antibodies used were 1:40 integrin mAb (mAb 1964, Chemicon) and α3 integrin mAb (Chemicon). After washing with immunofluorescence buffer, 1:100 secondary goat anti-mouse FITC-conjugated IgG (Zymed Laboratories, Inc., San Francisco, CA) was applied for 1 hour at room temperature. Nuclei were counterstained with 4’,6-diamidino-2-phenylindole (DAPI, Sigma). Control sections were stained with secondary antibodies only. The slides were mounted in fluoromount (Southern Biotechnology Associates, Inc., Birmingham, AL). The images were collected with Zeiss 410 LSM confocal microscope (Zeiss Pluar 63× oil objective lenses, Carl Zeiss Microimaging, Inc., Thornwood, NY). Images for figures were colored with Adobe Photoshop 7.0 Software.

**Immunofluorescence for activated extracellular signal-regulated kinase (ERK)** 1/2 was done using the same protocol above using the antibody ERK1/2 (pT202/pY204) (pT202/pY204) Phospho-Specific (BD Biosciences). Slides were analyzed under a Nikon Eclipse TE300 fluorescence microscope.

**Ki-67 proliferation index.** The proliferative rate of cells grown in three-dimensional cultures was assessed by Ki-67 immunostaining. Five-micrometer-thick frozen sections (see above) were fixed in methanol/acetone (1:1, v/v) for 10 minutes at room temperature, washed in 50 mmol/L PBS/glycine, blocked as described above, and incubated with a 1:50 dilution of Ki-67 mAb (Clon MB67, Neomarkers, Fremont, CA) in 10% goat serum immunofluorescence buffer plus 1:100 fab fragment, overnight at 4°C. After washing with immunofluorescence buffer, a 1:100 dilution of goat anti-mouse FITC conjugated IgG (Zymed) in immunofluorescence buffer containing 10% goat serum was applied for 40 minutes at room temperature. Slides were counterstained with DAPI and mounted in fluoromount (Southern...
biotinylated probes. Tissue sections were hybridized with 800 ng/ml of probe as previously described (3). Four of each fibrocystic, high-grade ductal carcinoma and invasive ductal carcinoma human breast tumor specimens were examined. Five of each normal, grade 2, and grade 3 human endometrial carcinoma was examined.

**Tumor formation in vivo.** A total of $5 \times 10^6$ MDA-MB-231pLXSN or MDA-MB-231pLD10 was injected s.c. into the flank region of nu/nu mice ($n=4$ mice/group, in each of two experiments; Charles River, Wilmington, MA). After 2 weeks, when tumors had developed, tumor volume was measured thrice a week for 6 weeks. Volume was calculated according to the following formula: $V (\text{mm}^3) = A (\text{mm}) \times B^2 (\text{mm}^2) / 2$, where $B$ is the smaller dimension.

Statistical analysis was done using Student’s $t$ distribution with significance reported when $P < 0.05$.

Procedures were done in accordance with regulations for the proper care and use of laboratory animals.

**Statistical analysis.** In all experiments, statistical analysis was done using Student’s $t$ distribution with significance reported when $P < 0.05$.

**Results**

**HoxD10 is lost during tumor progression.** In previous studies, we examined the expression of HoxD10 in blood vessels in normal and preinvasive breast tumors by **in situ** hybridization (3). During the course of these studies, we also noted diminished expression of HoxD10 in breast epithelial cells in high-grade ductal carcinoma **in situ** as compared with fibrocystic tissue. To fully evaluate expression of HoxD10 in normal, preinvasive, and invasive breast lesions, we did **in situ** hybridization on specimens of fibrocystic, ductal carcinoma **in situ**, and invasive human breast tissues. We observed strong expression of HoxD10 in premalignant epithelial cells (Fig. 1A), whereas expression of HoxD10 was relatively reduced in ductal carcinoma **in situ** (preinvasive breast tissue; Fig. 1B) and largely absent in invasive breast carcinomas (Fig. 1C). To study whether the progressive loss of HoxD10 expression was restricted to breast tumors, we subsequently did **in situ** hybridization on normal and increasingly tumorigenic endometrial tissue. Again, we observed positive hybridization of HoxD10 mRNA in nonmalignant endometrial epithelial cells (Fig. 1E) with progressive loss of HoxD10 expression in grade 2 endometrial tumors (Fig. 1F) and almost complete absence of HoxD10 expression in epithelial cells of grade 3 endometrial carcinoma (Fig. 1G). Figure 1D and H shows control hybridization using a HoxD10 sense riboprobe for both fibrocystic breast (Fig. 1D) and normal endometrium (Fig. 1H). These results show that HoxD10 expression decreases in both breast and endometrial epithelial cells as malignancy progresses, suggesting that its expression may have an important role in maintaining a nonmalignant phenotype.

**Sustained expression of HoxD10 impairs migration but not proliferation of tumor epithelial cells in conventional two-dimensional tissue culture.** We further analyzed expression of HoxD10 in at least eight invasive human breast cancer cell lines. Reverse transcription-PCR (RT-PCR) analysis revealed that HoxD10 was not expressed by MDA-MB-231, MDA-MB-453, BT-549, T47D, or SkBr3 cell lines (Fig. 2A). Although HoxD10 was also lacking in the invasive MDA-MB-468 and BT-20 breast cancer cell lines, low levels could be detected in the weakly malignant MDA-MB-436 cell line (not shown). In addition, HoxD10 was also not expressed in the cervical cancer cell line HeLa (Fig. 2A). In contrast, normal human microvascular endothelial cells continue to express HoxD10. To determine the effects of restoring HoxD10 expression on tumorigenic epithelial cells, we stably transduced both HeLa and the highly invasive HoxD10-negative breast epithelial tumor cell line MDA-MB-231 with a retroviral vector carrying the human HoxD10 cDNA (pLD10), a HoxD10 point mutant which does not bind DNA (pLD10 N-T), or the control vector (pLXSN). RT-PCR showed that whereas no endogenous expression of HoxD10 could be detected in control-transduced HeLa and MDA-MB-231 cells (HeLapLXSN and MDA-MB-231pLXSN), HoxD10 expression was abundantly expressed following selection of cells transduced with the HoxD10 retroviral vector (HeLapLD10, MDA-MB-231pLD10, and MDA-MB-231pLD10N-T; Fig. 2B).

Using the MTT assay to assess proliferation, we did not detect any significant differences between control and HoxD10-transduced cells either in MDA-MB-231 or HeLa cells (Fig. 2B). In addition, we also evaluated incorporation of BrdUrd in control and HoxD10-transfected cells but did not observe any significant differences (data not shown). We had previously observed that HoxD10 did not induce growth arrest in cultured endothelial cells, yet **in vivo** HoxD10 was a potent inhibitor of angiogenesis.

In tissue culture studies, we observed that HoxD10 impaired endothelial cell migration in response to basic fibroblast growth factor and vascular endothelial growth factor (3) and, therefore, we assessed migration in control or HoxD10-transduced tumor cells using modified Boyden chambers coated with type I collagen. HoxD10 significantly decreased migration (60%) of both HeLa and MDA-MB-231 cells compared with control cells (Fig. 2C). In contrast, a HoxD10 mutant, which does not bind DNA, did not inhibit migration of MDA-MB-231 cells, indicating that binding to DNA and subsequent effects on gene expression were required for HoxD10-mediated suppression of migration (Fig. 2C). To eliminate the possibility that changes in gene expression were altering the ability to adhere to the collagen matrix rather than influencing migration per se, we also evaluated adhesion to type I collagen but did not observe any significant differences in control or HoxD10-transfected MDA-231 or HeLa cells (data not shown).

Moreover, HoxD10 impaired migration in MDA-MB-231 cells to a similar degree as treatment with function-blocking antibodies against α3 integrin and uPAR, or with the MMP inhibitor GM6001 (Fig. 2D).

**HoxD10 induces tumorigenic epithelial cells to acquire an organized polarized phenotype when cultured in the 3DlrBM model.** Interactions between epithelial cells and the microenvironment, in particular the extracellular matrix, are critical for maintaining normal tissue architecture and differentiated function. When premalignant or invasive breast tumor cells are cultured in 3DlrBM, they exhibit distinct phenotypic features, which cannot be observed in conventional two-dimensional tissue culture plastic. Whereas tumor cells form large disorganized, nonpolarized structures, nonmalignant epithelial cells form polarized, organized structures reminiscent of acinar structures observed in the normal breast **in vivo** (28). We therefore cultured control and HoxD10-expressing MDA-MB-231 cells in 3DlrBM. In contrast to control-transduced MDA-MB-231 cells, which formed large, stellate disorganized clusters (Fig. 3A), MDA-MB-231 expressing HoxD10 cells formed small, compact structures similar to the acinar-like structures formed by nonmalignant breast cells (Fig. 3B; ref. 28). The percentage of organized colonies increased by 2-fold in MDA-MB-231pLD10 compared with control cells (Fig. 3C).
Subsequent immunofluorescent staining for \( \beta_4 \) integrin confirmed that this integrin was redistributed to the basolateral surface of HoxD10-transduced MDA-MB-231 but not control cells, cultured in 3DlrBM cultures, consistent with formation of polarized structures (Fig. 4A, top). However, neither expression of \( \beta_4 \) integrin mRNA nor protein changed between HoxD10-expressing cells and controls, as shown by RT-PCR (Fig. 4B) and Western blot analysis (Fig. 4C), respectively. Our previous studies showed that in endothelial cells, HoxD10 impaired expression of \( \alpha_3 \) integrin, uPAR, and MMP-14, all genes known to promote cell migration and invasion in both endothelial and breast tumor cells (23–25). To determine whether similar target genes were being suppressed by HoxD10 in breast tumor cells, we did immunofluorescence and RT-PCR analysis on control or HoxD10-expressing cells in 3DlrBM. Immunofluorescence staining of MDA-MB-231 revealed that \( \alpha_3 \) integrin expression was redistributed and reduced in HoxD10-expressing MDA-MB-231 cells compared with control cells (Fig. 4A, bottom). In contrast to \( \beta_4 \) integrin, however, \( \alpha_3 \) integrin mRNA and protein levels in MDA-231-pLD10 compared with control cells were significantly reduced as shown by RT-PCR analysis (Fig. 4B) and Western blot analysis (Fig. 4C). However, mRNA levels of uPAR and MMP-14 did not significantly change in the presence or absence of HoxD10 (Fig. 4B). Figure 4B (bottom) shows expression of HoxD10 in transduced MDA-MB-231 compared with control cells as determined by RT-PCR analysis.

Previous studies using the 3DlrBM assay also noted that when tumor cells were reverted to more organized, polarized structures, they also underwent growth arrest accompanied by a reduction in mitogen-activated protein kinase (MAPK) signaling (29). We therefore did immunofluorescent staining for phospho-ERK1/2 in control or HoxD10-expressing MDA-MB-231 cells in 3DlrBM and observed a marked reduction in intensity of phospho-ERK1/2 in HoxD10 expressing cells (Fig. 5A). Consistent with the formation of organized polarized acini, and reduced phospho-ERK1/2, MDA-MB-231 cells expressing HoxD10 showed a significantly reduced percentage of Ki-67–positive cells, indicating induction of growth arrest (Fig. 5B and C).
Thus, restoring HoxD10 not only impairs migration but also promotes cell-cell interactions and cell-extracellular matrix interactions and induces growth arrest, which results in the formation of polarized acinar-like structures. Together these results suggest that HoxD10 may function as a tumor suppressor and prevent breast cancer progression in vivo.

**HoxD10 inhibits tumor growth in vivo.** To determine the effects of HoxD10 on breast tumor growth in vivo, we inoculated MDA-MB-231pLXSN control and MDA-MB-231pLD10 into nude mice. Cells were injected s.c. into female nude mice and tumor volume was measured weekly for 6 weeks. Throughout the experiment, tumor volume was significantly smaller in mice that received MDA-MB-231pLD10 cells compared with the control group (Fig. 6). Furthermore, three of eight mice that received HoxD10-expressing cells were free of any measurable tumors 6 weeks after cell injection. Control mice were euthanized when tumor volume reached about 1 cm³, 6 weeks after cell injection, whereas the HoxD10 tumor-free mice were monitored for another 10 weeks and remained tumor-free. Thus, HoxD10 is a potent inhibitor of tumor growth in vivo.

**Discussion**

We show that expression of HoxD10 is progressively lost in both breast and endometrial tissue with increasing tumorigenicity. However, if expression of HoxD10 is restored in malignant breast tumor cells using retroviral gene transfer, cell migration is impaired, a normal polarized, acinar morphology in three-dimensional cultures is restored, and tumor growth in vivo is inhibited. Taken together, our results suggest that HoxD10 normally functions as a tumor suppressor in breast tissue.

Our results, together with an earlier study showing loss of HoxD10 expression in endometrial tumors relative to normal tissue (16), supports a hypothesis that HoxD10 may act to stabilize or maintain a differentiated phenotype in both breast and endometrial epithelial cells. Previous studies examining targeted disruption of HoxD10 showed that four of six females initially failed to undergo lactation (30). Subsequent analysis of these mice revealed that whereas alveolar development progressed normally through pregnancy, these mice failed in whole or in part to produce milk and expand their alveoli, a hallmark of functionally differentiated mammary epithelial cells (19). It is also worth noting that constitutive expression of the paralogous HoxA10 in BT20 cells, another aggressive human breast cancer cell line, also reduces their invasion through Matrigel (31). In addition, HoxA10 induces growth arrest in myelomonocytic cells and thus might also function as a tumor suppressor (32).

A recent study by Cantile et al. (33) did not detect expression of HoxD10 in normal human breast, but instead observed HoxD10...
expression in 7 of 14 breast cancer biopsies. The reason for this discrepancy is not clear. However, as their analysis was based on RT-PCR using total tissue RNA, it is possible that activated stromal cells may contribute to the increase in HoxD10 expression observed in tumor tissue, whereas the proportion of epithelial cells in normal tissue, where we detected strong HoxD10 expression, comprises only a minor proportion of total breast tissue RNA. Moreover, while this article was under review, a more recent study using real-time PCR reported significantly lower levels of HoxD10 in human invasive ductal breast cancer tissue compared with normal breast (34), further supporting our in situ hybridization data.

Our current results also support our previous observations for the role of HoxD10 in inhibiting migration in vascular endothelial cells (3) and previous studies showing that HoxD10 is strongly expressed in differentiated breast epithelial cells (19). It is of interest that we could not detect significant levels of HoxD10 in any cultured epithelial cells, despite the strong expression we observed in normal epithelium in vivo. These findings suggest that the normal breast microenvironment plays a key role in maintaining HoxD10 expression. However, it is not clear what changes associated within the tumor microenvironment lead to the loss of HoxD10 expression.

Our expression analysis data in three-dimensional cultures indicate that HoxD10 suppresses expression of the α3 integrin subunit, member of the heterodimeric α3β1 integrin, which recognizes a variety of extracellular matrix proteins including laminin-5 and collagen, and mediates both cell-extracellular matrix and cell-cell interactions. It has been reported in various tumors, including breast cancer, that the aberrant expression of α3β1 integrin is associated with a high invasive and metastatic potential (23, 26, 35). How α3 integrin disrupts normal interactions with the basement membrane is not entirely clear but α3 integrin also functions as a receptor for proteolyzed laminin fragments, generated by high proteolytic activity of invasive breast tissue (36), which in turn would promote migration and invasion.

Our results suggest that HoxD10 inhibits migration by reducing expression of α3 integrin. This is consistent with the migration assay results showing that function-blocking antibody to α3 integrin inhibited migration of MDA-MB-231 cells to the same extent as HoxD10. Whether α3 integrin is a direct target for HoxD10 is not known. The human promoter region for α3 integrin has not yet been identified, but the mouse α3 integrin promoter contains at least two Hox binding recognition sites (TNAT/C; ref. 37). Chromatin immunoprecipitation analysis is currently under way in our laboratory to determine whether the promoter of the α3 integrin is a direct or secondary target for HoxD10.

We previously showed that HoxD10 inhibits expression of uPAR, MMP-14, and α3 integrin in endothelial cells (3). Although HoxD10 inhibits expression of α3 integrin in MDA-MB-231 cells, we did not detect differences in uPAR or MMP-14 mRNA expression levels between control and HoxD10-expressing cells, suggesting these genes may not be direct transcriptional targets of HoxD10 in MDA-MB-231 cells. It is also worth noting that α3 integrin is required for proper uPAR localization and proteolytic and signaling functions (38, 39). Thus, inhibition of α3 integrin expression may lead to decreased plasminogen activity and lead to a reduction in migration.

In a recent article, Wang et al. (40) also reverted MDA-MB-231 breast tumor cells to a normal phenotype using the 3DlrBM model. Whereas inhibition of epidermal growth factor receptor, β1 integrin, phosphatidylinositol 3-kinase, or MAPK alone was not sufficient...
to revert MDA-MB-231 cells, blocking β1 integrin or expressing E-cadherin in combination with inhibition of phosphatidylinositol 3-kinase or MAPK effectively reverted the cells to a nonmalignant phenotype. Our results show that restoring HoxD10 alone was sufficient to phenotypically revert the malignant phenotype of MDA-MB-231, reduce signaling through the MAPK kinase pathway, and induce growth arrest. Indeed, whereas the levels of total ERK protein in both control and HoxD10 expressing MDA-MB-231 cells were similar, only the activity of ERK was reduced with HoxD10. Moreover, following transfection with HoxD10, we did not detect any reexpression of E-cadherin or changes in the levels of β3 and β4 integrins in MDA-MB-231 cells which have previously been linked to reduced MAPK signaling in breast tumor cells (40, 41). Similarly, using a combination of treatments to induce polarity and morphologic reversion in MDA-MB-231 cells, Wang et al. (40) did not observe any reexpression of E-cadherin. Whether other cadherins are induced and contribute to the polarized phenotype acquired by MDA-MB-231 following transfection with HoxD10 or treatment with a combination of inhibitors remains to be established.

Nonetheless, it is likely that the suppression of MAPK activity by HoxD10 occurs indirectly by promoting proper interactions with the basement membrane, perhaps via attenuation of α3 integrin expression, which otherwise facilitates degradation of the extracellular matrix and cellular invasion (23, 26). Although signaling via α3 integrin has not been previously studied in breast tumor cells, recent studies show that signaling through this laminin receptor leads to high levels of ERK activity in dermal epithelial cells (42). Nonetheless, our findings that restoring HoxD10 expression alone was sufficient to revert the phenotype of the aggressive MDA-MB-231 cells further supports the notion that HoxD10 affects key regulatory pathways and is consistent with a role for Hox factors as master regulatory genes.

Normal tissue architecture depends on proper cell-cell and cell-extracellular matrix interactions, and disruption of these interactions not only results in loss of tissue structure but also contributes to tumor progression. Thus, as tumors exhibit gross defects in normal tissue patterning, it is not surprising that alterations in expression of Hox genes, which direct normal tissue patterning, are common in many different solid tumors (for review, see refs. 19, 43). Moreover, using standard two-dimensional culture
conditions, where most cells exhibit a similar monolayer morphology and proliferation rate, it may not be possible for patterning genes to fully exert their influence in directing more complex tissue-type morphologies which they readily induce in vivo. Indeed, whereas previous studies have noted that loss of HoxA5 leads to impaired differentiation of gut and stomach epithelial cells, HoxA5 expression is primarily restricted to the adjacent mesenchyme and the subsequent effect on epithelial differentiation is derived by loss of inductive signals (44, 45).

The utility of using the 3DlrBM approach to recapitulate phenotypic aspects of breast tumor cells in vivo has elegantly been shown by Bissell and others (28, 41, 46–51). Our results further emphasize that three-dimensional culture models or in vivo assays are required to fully appreciate the effect of morphoregulatory genes including HoxD10 on tissue patterning and morphology of breast epithelial cells. Moreover, we had previously observed that whereas HoxD10 does not induce growth arrest in endothelial cells when cultured in conventional two-dimensional tissue culture methods, in vivo HoxD10 was a potent inhibitor of angiogenesis (3). Similarly, whereas HoxD10 also does not affect proliferation of breast tumor cells in two-dimensional cultures, in three-dimensional cultures using lrBM, or in vivo, HoxD10 leads to growth arrest. In previous studies, we observed that serum withdrawal was not sufficient to induce growth arrest in nonmalignant mammary epithelial cells in two-dimensional culture, but culturing in 3DrBM led to suppressed expression of cyclin D1 and growth arrest (29). Together these findings suggest that HoxD10 acts to restore the tumor cells ability to respond to cues from the extracellular matrix and subsequently undergo growth arrest.

Interestingly, previous studies have shown that Hox genes located near the opposite 3′ end of the clusters are linked with oncogenic transformation and increased invasion. Forced expression of HoxA1 in MCF-10A cells results in oncogenic transformation and the development of a rapidly growing carcinoma in vivo (22). HoxD3 induces expression of metastasis-related genes in human lung cancer cells (52, 53). Results from our laboratory show that HoxD3 also promotes expression of several genes which promote invasion in endothelial cells including integrins αvβ3, α5β1, and uPA (5, 54),

Figure 6. HoxD10 inhibits tumor growth in vivo. Control or HoxD10-expressing cells (5 × 10⁶) were injected s.c. into nude mice at day 0. After 14 days, when tumors developed, tumor volume was measured for 6 weeks. Points, means (n = 8 mice per group); bars, SD. **, P < 0.01. P values calculated using Student’s t test.

Figure 5. HoxD10 induces growth arrest in breast tumor cells cultured in 3DrBM. A, immunofluorescence staining for phosphorylated ERK1/2 and DAPI nuclear staining in control (MDA-MB-231pLXSN) and HoxD10 (MDA-MB-231pLD10) expressing cells. Original magnification, ×20; bar, 10 μm. B, immunofluorescence staining for Ki-67 and DAPI. Original magnification, ×20; bar, 10 μm. C, quantitative analysis of Ki-67 labeling index as determined by counting nuclei stained with DAPI in at least 300 cells and thereafter scoring Ki-67–positive cells as a percentage of the total cell number. Columns, means; bars, SD. **, P < 0.01, compared with controls; P values calculated using Student’s t test. Cultures were analyzed after 4 days in 3DrBM.
when overexpressed in vivo, HoxD3 promotes a hemangioma-like proliferation of blood vessels (5, 55). Taken together, these results are consistent with the distinct roles predicted for 5′ and 3′ Hox genes based on their nonoverlapping expression patterns during development and with the increasing expression of 5′ Hox genes in differentiating cells (56, 57).

Acknowledgments

References

Homeobox D10 Induces Phenotypic Reversion of Breast Tumor Cells in a Three-Dimensional Culture Model

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