Human Growth Hormone-regulated HOXA1 Is a Human Mammary Epithelial Oncogene*

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Increased mammary epithelial expression of the human growth hormone (hGH) gene is associated with the acquisition of pathological proliferation. We report here that autocrine hGH production by human mammary carcinoma cells increased the expression and transcriptional activity of the homeobox domain containing protein HOXA1. Forced expression of HOXA1 in human mammary carcinoma cells resulted in increased total cell number primarily by the promotion of cell survival mediated by the transcriptional up-regulation of Bcl-2. HOXA1 also abrogated the apoptotic response of mammary carcinoma cells to doxorubicin. Forced expression of HOXA1 in mammary carcinoma cells, in a Bcl-2-dependent manner, resulted in dramatic enhancement of anchorage-independent proliferation and colony formation in soft agar. Finally, forced expression of HOXA1 was sufficient to result in the oncogenic transformation of immortalized human mammary epithelial cells with aggressive in vivo tumor formation. Herein, we have therefore provided a molecular mechanism by which autocrine hGH stimulation of mammary epithelial cells may result in oncogenic transformation.

Signal transduction pathways that regulate mammary epithelial cell proliferation, differentiation, apoptosis, and transformation have been delineated and utilized as therapeutic targets for the treatment of mammary gland neoplasia (1, 2). Examples of such include selective estrogen modulators (3) and hereceptin (4) targeting estrogen receptor and HER-2 pathways, respectively. However, many carcinomas of the mammary gland neither express the estrogen receptor (5) nor overexpress HER-2 (6) and are, therefore, not responsive to these specific therapeutic strategies. Clinical data for the treatment of cancer has clearly demonstrated the superiority of combinatorial therapy over a single-agent approach (7). Delineation, characterization, and development of therapeutic regimes targeting novel signaling pathways involved in oncogenic transformation of mammary epithelial cells are therefore required.

Growth hormone (GH)† is obligatory for normal pubertal mammary gland development (8). Specifically, GH acts on both the mammary stromal and epithelial components to result in ductal elongation and the differentiation of ductal epithelia into terminal end buds (9, 10). Expression of the hGH transgene in mice results in precocious development of the mammary gland (11, 12) and the development of neoplasia (12). Conversely, spontaneous or experimentally engineered functional deficiency of GH (13–16) results in severely impaired mammary gland development and virtual resistance to the spontaneous development of hyperplastic alveolar nodules (13) and chemically induced mammary carcinogenesis (14, 16). Similarly, in a primate model, hGH administration results in marked hyperplasia of the mammary gland with an increased epithelial proliferation index (17). Thus, the somatotropic axis represents one potential and unutilized approach for novel therapeutic approaches to the treatment of mammary epithelial neoplasia.

The hGH gene is also expressed in epithelial cells of the normal human mammary gland (18). Increased epithelial expression of the hGH gene is associated with the acquisition of pathological proliferation, and the highest level of hGH gene expression is observed in metastatic mammary carcinoma cells (18). hGH receptor gene expression per mammary epithelial cell remains constant throughout the process of neoplastic progression (19), and therefore, alterations in the local concentration of ligand are likely to be pivotal in determining the effects of hGH on mammary epithelial cell behavior. We have recently generated a model system to study the role of autocrine-produced hGH in mammary carcinoma by stable transfection of either the hGH gene or a translation-deficient hGH gene into mammary carcinoma cells (20). The autocrine production of hGH by mammary carcinoma cells results in a hyperproliferative state with marked synergism between trophic agents such as insulin-like growth factor 1 (20). The increase in mammary carcinoma cell number as a consequence of autocrine production of hGH is a result of both increased mitogenesis and decreased apoptosis (21). Thus, autocrine production of hGH by mammary carcinoma cells may direct mammary carcinoma cell behavior to impact on the final clinical prognosis, and therefore, systematic analysis of the relevant mechanistic features by which it exerts its cellular effects is required.

One major mechanism by which GH affects cellular and somatic function is by regulating the level of specific mRNA species (22). We have previously utilized cDNA microarray analyses to identify genes regulated by autocrine production of hGH in human mammary carcinoma cells (MCF-7) (23). One gene demonstrated to be up-regulated by autocrine hGH in MCF-7 cells was the homeobox containing transcription factor HOXA1 (23). Homeobox-containing genes are a family of genes encoding transcription factors that possess pivotal roles in development (24). For example, HOXA1 has been demonstrated to be required for vertebrate hindbrain segmentation (25). Sev-
eral reports also suggest the involvement of homeobox-containing genes in the control of cell proliferation and, when dysregulated, in oncogenesis (26, 27). Moreover, alterations of HOX gene expression have been detected in a variety of human tumors (26–28), including those of the mammary gland (29, 30). Accordingly, HOXA1 has been detected in carcinoma of the mammary gland in the mouse but not in normal mammary tissue (13). HOXA1 expression has also been detected in neoplastic lesions of the human mammary gland (32).

Herein we provide a molecular mechanism by which hGH stimulation of human mammary epithelial cells results in oncogenic transformation. hGH-regulated HOXA1 increases mammary epithelial cell number primarily by prevention of apoptotic cell death in a Bcl-2-dependent manner, with resultant anchorage-independent growth and tumor formation in vivo. Functional antagonism of hGH, and the molecular pathways it utilizes, will therefore constitute novel adjunct therapeutic approaches to the treatment of mammary gland neoplasia.

EXPERIMENTAL PROCEDURES

Cell Culture—The MCF-7 cell line and MCF-10A cell line were obtained from the ATCC. MCF-7 cells and derivatives (see below) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. MCF-10A cells and derivatives (see below) were cultured in Dulbecco’s modified Eagle’s medium/F-12 medium (Invitrogen, Carlsbad, CA) supplemented with 5% horse serum (Invitrogen) plus 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 0.25 μg/ml ampicillin B, 100 ng/ml cholera toxin, 20 ng/ml epidermal growth factor (Upstate Biotechnology, Lake Placid, NY), 0.5 μg/ml hydrocortisone (Calbiochem, La Jolla, CA), and 10 μg/ml insulin. Fetal bovine serum was purchased from HyClone Laboratories (Logan, UT), and all other cell culture materials were obtained from Sigma Chemical Co. (St. Louis, MO).

The MCF-7 cell line was stably transfected with an expression plasmid containing the wild-type hGH gene (pMT-hGH) (33) under the control of the metallothionein 1α promoter (designated MCF7-hGH) (20). For control purposes the ATG start site in pMT-hGH was disabled via a mutation to TTG generated by standard techniques (pMT-MUT) (35), and MCF-7 cells stably transfected with this plasmid were designated MCF7-MUT. A detailed description of the characterization of these cell lines has been published previously (20). MCF-7 and MCF-10A cell lines were stably transfected with an HOXA1 expression plasmid (pSG5-HOXA1) and pcDNA3 empty vector in a ratio of 5:1 by use of lipofection transfection reagent obtained from Qiagen (Hilden, Germany) according to the manufacturer’s instruction. pSG5-HOXA1 is the generous gift of Dr. Vincenzo Zappavigna (Milano, Italy). Positive transfectants were selected in 800 μg/ml G418 (Calbiochem) in the appropriate culture medium for the respective cell lines. Individual colonies were selected to determine the HOXA1 expression level. Cell lines were established as MCF7-HOXA1 and MCF10A-HOXA1, respectively, by pooling five individual colonies. Likewise, MCF-7 and MCF-7-10A cells, stably transfected with vector, were also established (MCF7-Veector and MCF10A-Veector) as control.

Reverse Transcriptase-PCR—Expression of total RNA and the RT-PCR assay were performed as described previously (23). To compare the PCR products semi-quantitatively, 15–40 cycles of PCR (annealing temperature was also varied to determine linearity of the PCR amplification, and the amplified β-actin cDNA served as an internal control for cDNA quantity and quality. All RNA samples were treated with DNase I to avoid genomic DNA contamination.

The sequences of the oligonucleotide primers used for RT-PCR are as follows: HOXA1 (sense), 5’-GGGAAAAATTGGAGAAGTTACCGG-3’; HOXA1 (antisense), 5’-CAGTATCTCGGGCCCTGC-3’; β-actin (sense), 5’-AGTATGATGCCGGCTCTGC-3’; β-actin (antisense), 5’-TCCGTCGTTGAGATCTTGA-3’; Bcl-2 (sense), 5’-TGGCCAATGACCGCTCTTAC-3’; Bcl-2 (antisense), 5’-TGCACCATGGACGACCCTTAC-3’; Bcl-xL (sense), 5’-TGGCCAATGACCGCTCTTAC-3’; Bcl-xL (antisense), 5’-TGGCCAATGACCGCTCTTAC-3’.

 Luciferase Reporter Assay for EphA2-42B and Bcl-2 Promoter Constructs—Cells were cultured to 40–60% confluence in six-well plates. Transfections were performed by use of Effectene transfection reagent obtained from Qiagen (Hilden, Germany) according to the manufacturer’s instruction. For incorporation of BrdUrd as described (21). For incorporation of BrdUrd, subconfluent cells were pulse-labeled with 20 μM BrdUrd for 30 min, washed twice with PBS, and fixed in cold 70% ethanol for 30 min. BrdUrd detection was performed by use of the BrdUrd staining kit from Zymed Laboratories Inc. (San Francisco, CA). Immunolabeling was visualized by ECL detection kit from Amersham Biosciences (Little Chalfont, UK) according to the manufacturer’s instruction.

5’-Bromo-2’-deoxyuridine Incorporation Assay—Mitogenesis was directly assayed by measuring the incorporation of BrdUrd as described (21). For incorporation of BrdUrd, subconfluent cells were pulse-labeled with 20 μM BrdUrd for 30 min, washed twice with PBS, and fixed in cold 70% ethanol for 30 min. BrdUrd detection was performed by use of the BrdUrd staining kit from Zymed Laboratories Inc. (San Francisco, CA) according to the manufacturer’s instruction. A total population of over three times 300 cells was analyzed in several arbitrarily chosen microscopic fields to determine the BrdUrd labeling index (percentage of cells synthesizing DNA).

Determination of Anchorage-dependent and-independent Growth of Transfected Cells—Cells (5 × 104) were seeded into six-well plates in monolayers in serum-free media or in serum-free media supplemented with either 50 ng hGH or 10% FBS. After 48 h, cells were trypsinized with 0.5% trypsin, and the cell number was determined using a hemocytometer. Cells (5 × 105) in suspension culture were grown in 30-mm plastic bacteriological dishes (Sterill, Teddington, United Kingdom). Cells were harvested and seeded as for monolayers. For soft agar colony formation assay, MCF-7 cell and its derived cell lines were cultured in six-well plates first covered with an agar layer (RPMI 1640 with 0.5% agar and 10% FBS). The middle layer contained 5 × 105 cells in RPMI 1640 with 0.35% agar and 10% FBS with or without 50 ng hGH or Bcl-2 inhibitor. Medium was added as the top layer to prevent drying of the agarose gels. The MCF10A-Veector and MCF10A-HOXA1 cell soft agar colony formation assay were processed the same as above, except that the medium was changed to Dulbecco’s modified Eagle’s medium/F-12 containing appropriate components as described, and 105 cells were seeded to the middle layer of the soft agar. The plates were incubated for 9 days (for MCF-7 cells) or 14 days (for MCF10A cells), after which the cultures were inspected and photographed.

Measurement of Apoptosis—Apoptotic cell death was measured by fluorescent microscopic analysis of DNA cell staining patterns with Hoechst 33258 from Sigma Chemical Co. (St. Louis, MO) (35). Cells were trypsinized with 0.5% trypsin and washed twice with serum-free medium. The cells were then seeded to glass cover slips in six-well plates and incubated in serum-free medium with or without 50 ng hGH, 10% FBS, doxorubicin, Bcl-2 inhibitor (Calbiochem), or Bcl-2 antisense oligonucleotide (Calbiochem) as indicated. After a culture period of 24 h, the cells were fixed in 4% paraformaldehyde in PBS (pH 7.4) and stained with the karyophilic dye Hoechst 33258 (20 μg/ml) for 10 min at 37°C. After washing with PBS, the sample was examined under a UV-visible fluorescence microscope (Zeiss Axioplan). Apoptotic cells were distinguished from viable cells by their nuclear morphology characterized by nuclear condensation and fragmentation as well as the higher intensity of the blue fluorescence of the nuclei. For statistical analysis, three times 300 cells were counted in each of five microscope fields at 400 magnification.

Use of Bcl-2 Antisense Oligonucleotide to Deplete Cellular Bcl-2—Cells were Cultured for 24 h in serum-free medium and then transfected with 800 nm oligonucleotide (Calbiochem) in 8 h in DOTAP with a final concentration of 10 μM as previously described (33). Bcl-2 (sense), 5’-CCCTTGCGTGACCCCTCT-3’; Bcl-2 (antisense), 5’-TCTCCCTTCAGTCTCC-3’.

 Xenograft Analyses—Severe combined immunodeficient (SCID) 4-week-old mice were purchased from the animal holding facility of the National University of Singapore and acclimated for 7–10 days. For subcutaneous implantation, 5 × 106 control MCF10A-Veector or
MCF10A-HOXA1 cells were suspended in 100 μl of PBS or Matrigel and injected into the first mammary (axillary) fat pad of SCID mice, which simultaneously received a 60-day release pellet containing 0.72 mg of E2 (Innovative Research of America, Toledo, OH). At necropsy, primary tumors and all organs were evaluated macroscopically for the presence of tumors. Tissue samples of the primary tumor and organs were fixed in 4% paraformaldehyde in PBS (pH 7.4) and embedded in paraffin. Tissue sections of the tumors were stained with H&E to assess morphology. Total RNA extraction and RT-PCR were performed by use of fresh lung and liver tissue to detect possible metastasis of the tumors.

**Statistics**—All experiments were repeated at least three to five times. All numerical data are expressed as mean ± S.E., and the data were analyzed using Instat 3.0 from GraphPad Software Inc.

**RESULTS**

**Autocrine hGH Stimulation of Mammary Carcinoma Cells Increases HOXA1 mRNA, Protein, and Transcriptional Activity**—By use of cDNA microarray analysis we had previously identified HOXA1 as an autocrine hGH-regulated gene in human mammary carcinoma cells (23). To verify the autocrine hGH-dependent up-regulation of HOXA1 mRNA observed by cDNA array screening, we first examined the level of HOXA1 mRNA in MCF7-MUT and MCF7-hGH cells by semi-quantitative RT-PCR. The validation that the conditions of RT-PCR used here yielded semi-quantitative estimates of mRNA is described under “Experimental Procedures” and has been utilized previously by us (23, 36). One amplified fragment of the predicted size (359 bp) appropriate for HOXA1 mRNA was detected in MCF7-MUT and MCF7-hGH cells (Fig. 1A). Autocrine production of hGH by MCF7-hGH cells resulted in an increased level of HOXA1 mRNA in comparison to MCF7-MUT cells. Exogenous hGH and FBS also increased HOXA1 mRNA in MCF7-MUT cells and potentiated the increased level of HOXA1 mRNA observed in MCF7-hGH cells due to autocrine production of hGH. The level of β-actin mRNA did not differ between the two cell lines under the different treatments and was used as a control for RNA quality (Fig. 1A).

HOXA1 cDNA transfected into MCF-7 cells results in the appearance of two distinct protein species of 33 and 35 kDa (for example see Fig. 2B) as expected (37). To determine if the autocrine hGH-stimulated increase in HOXA1 mRNA also resulted in increased HOXA1 protein, we examined the level of HOXA1 protein in nuclear extracts collected from MCF7-MUT and MCF7-hGH cells by Western blot analysis. Both the 33- and the 35-kDa forms of HOXA1 were detected in nuclear extracts from MCF7-MUT and MCF7-hGH cells (Fig. 1B) with increased HOXA1 protein observed in MCF7-hGH cells. FBS also increased the level of HOXA1 in MCF7-MUT cells and potentiated the level of HOXA1 in MCF7-hGH cells (Fig. 1B). Exogenous hGH did not stimulate an increase in HOXA1 protein in MCF7-MUT cells and only minimally enhanced the level in MCF7-hGH cells.

To determine if the autocrine hGH-stimulated increase in HOXA1 protein also resulted in increased HOXA1-mediated transcription, we examined luciferase reporter activity from the EphA2-r2B enhancer, which contains four HOX-PBX binding sites (38). Autocrine production of hGH by MCF7-hGH cells resulted in increased luciferase activity from the EphA2-r2B enhancer compared with MCF7-MUT cells (Fig. 1C) indicative of increased HOXA1 transcriptional activity. Transient transfection of HOXA1 cDNA increased HOXA1 transcriptional activity in MCF7-MUT cells and dramatically enhanced the already greater HOXA1 transcriptional response in MCF7-hGH cells. Transient transfection of MCF7-MUT cells with PBX1 cDNA was without effect, whereas transient transfection of cDNA for the HOX binding partner PBX1 dramatically increased HOXA1-mediated transcription in MCF7-hGH cells. Transient transfection of MCF7-MUT cells with both HOXA1 and PBX1 cDNAs did, however, result in a robust transcriptional response through the EphA2-r2B enhancer. Transient transfection of MCF7-hGH cells with both PBX1 and HOXA1 cDNAs synergistically increased HOXA1-mediated transcription above the enhancement observed with either HOXA1 or PBX1 alone (Fig. 1C). Thus, autocrine hGH production by mammary carcinoma cells results in increased HOXA1-mediated transcriptional activity.

**Forced Expression of HOXA1 in Human Mammary Carcinoma Cells Results in Increased Total Cell Number**—To determine the functional consequences of the autocrine hGH-stimulated increased HOXA1 expression in mammary carcinoma cells we generated a mammary carcinoma cell line (MCF-7) with increased expression of HOXA1 by stable transfection of HOXA1 cDNA (termed MCF7-HOXA1). Forced expression of HOXA1 mRNA in MCF7-HOXA1 cells compared with vector transfected (MCF7-VECTOR) cells was verified by RT-PCR analysis (Fig. 2A), and MCF7-HOXA1 also demonstrated higher levels of HOXA1 protein compared with MCF7-VECTOR cells (Fig. 2B). Analysis of HOXA1 transcriptional activity by use of the EphA2-r2B enhancer demonstrated increased HOXA1-mediated transcriptional activity in MCF7-HOXA1 cells compared with MCF7-VECTOR cells under serum-free conditions or when stimulated with exogenous hGH or FBS (Fig. 2C). Transient transfection of cDNA for the HOXA1 binding partner PBX1 in MCF7-VECTOR cells did not alter the level of HOXA1-mediated transcriptional activity under serum-free conditions or exogenous hGH stimulation and only slightly enhanced HOXA1-mediated transcriptional activity of MCF7-
The D family of cyclins is pivotal to initiate progression through the G1 phase of the cell cycle (39). Cyclin D1 is the predominant member of this family expressed in mammary gland (40), and we have previously demonstrated its requirement for autocrine hGH-stimulated mammary carcinoma cell cycle progression (36). We therefore examined the level of cyclin D1 protein in MCF7-VECTOR compared with MCF7-HOXA1 cells. The level of cyclin D1 protein was increased in MCF7-HOXA1 compared with MCF7-VECTOR cells (Fig. 3D) under serum-free conditions. Both exogenous hGH and FBS increased cyclinD1 protein in MCF7-VECTOR cells and accentuated the increase in cyclinD1 observed upon forced expression of HOXA1 in MCF7-HOXA1 cells (Fig. 3D). We consequently also examined the expression of the cdk inhibitor p21\(^{waf1/cip1}\) in MCF7-VECTOR and MCF7-HOXA1 cells. MCF7-HOXA1 cells exhibited a marked decrease in p21\(^{waf1/cip1}\) protein in comparison to MCF7-VECTOR cells (Fig. 3D). We next examined the protein level of the cyclin-dependent kinase inhibitor p27\(^{kip1}\). Decreased expression of p27\(^{kip1}\) is associated with G\(_1\)/S phase transition (41). The level of p27\(^{kip1}\) was not altered between MCF7-VECTOR and MCF7-HOXA1 cells (Fig. 3D) under the conditions studied. Equal loading of the cell extracts was verified by reprobing the stripped membrane for β-actin (Fig. 3D). To determine the effects of forced expression of HOXA1 on cell cycle progression in MCF7-VECTOR and MCF7-HOXA1 cells, we examined the nuclear incorporation of 5′-bromo-2′-deoxyuridine in these cells. Although MCF7-HOXA1 cells exhibited a significantly higher percentage of nuclear BrdUrd incorporation compared with MCF7-VECTOR cells (Fig. 3D) the differences were small and could not account for the observed differences in total cell number between MCF7-VECTOR and MCF7-HOXA1 cells.

We therefore examined the effect of forced expression of HOXA1 on a variety of proteins involved in the apoptotic process. The level of Bax, Bak, Bel-xl, Fas, and FADD did not differ between MCF7-VECTOR and MCF7-HOXA1 cells under serum-free conditions nor with stimulation by either exogenous hGH or FBS (Fig. 3E). The level of p53 did not differ between MCF7-VECTOR and MCF7-HOXA1 cells in serum-free media nor by stimulation with exogenous hGH but was dramatically reduced in both cell lines in the presence of FBS. Interestingly, however, the level of Bcl-2 was dramatically increased in MCF7-HOXA1 cells compared with MCF7-VECTOR cells in serum-free media, and the level of Bcl-2 was even further increased in MCF7-HOXA1 cells in the presence of FBS. Apoptotic cell death was also dramatically reduced in MCF7-HOXA1 cells compared with MCF7-VECTOR cells in serum-free conditions (Fig. 3C). Exogenous hGH slightly reduced apoptotic cell death in both cell lines. FBS functioned as a powerful survival stimulus for both cell lines, although apoptosis was still less in MCF7-HOXA1 cells compared with MCF7-VECTOR cells (Fig. 3C).

**HOXA1 Expression in Human Mammary Carcinoma Cells Prevents Apoptotic Cell Death in a Bcl-2-dependent Manner**—Forced expression of HOXA1 in mammary carcinoma cells resulted in decreased apoptotic cell death associated with a specific increase in Bcl-2 protein. To determine if HOXA1 regulated Bcl-2 at the transcriptional level, we first examined the effect of forced expression of HOXA1 in mammary carcinoma cells on the level of Bcl-2 mRNA. Increased Bcl-2 mRNA was observed in MCF7-HOXA1 in comparison to MCF7-VECTOR cells in serum-free media (Fig. 4A). FBS stimulation of MCF7-VECTOR cells also increased Bcl-2 mRNA in comparison to the serum-free state, and FBS enhanced the level of Bcl-2 mRNA in MCF7-HOXA1 cells. Examination of Bcl-2 P1 promoter activity demonstrated that the effect of forced expres-
sion of HOXA1 on Bcl-2 mRNA expression was mediated at the transcriptional level. Thus, Bcl-2 P1 promoter activity was significantly higher in MCF7-HOXA1 cells in comparison to MCF7-VECTOR cells in serum-free conditions (Fig. 4B). Exogenous hGH was without effect on Bcl-2 P1 promoter activity, but FBS stimulated Bcl-2 gene transcription in MCF7-VECTOR cells and dramatically enhanced Bcl-2 P1 promoter activity in MCF7-HOXA1 cells. Thus HOXA1 regulates Bcl-2 at the transcriptional level.

To determine if Bcl-2 was responsible for the dramatic decrease in apoptosis as a consequence of forced expression of HOXA1 we prevented expression of Bcl-2 in MCF7-VECTOR and MCF7-HOXA1 cells by use of antisense oligonucleotides (42) as observed in Fig. 4C. Transfection of antisense oligonucleotides to Bcl-2 prevented the HOXA1-stimulated expression of Bcl-2 in MCF7-HOXA1 cells, whereas sense control oligonucleotides did not significantly affect the level of Bcl-2 (Fig. 4C).

Transfection of Bcl-2 antisense oligonucleotides in MCF7-HOXA1 increased apoptotic cell death above that in control or sense oligonucleotide-transfected MCF7-VECTOR cells (Fig. 4D), and Bcl-2 antisense also increased apoptotic cell death in MCF7-VECTOR cells. To further verify the Bcl-2 dependence of the survival effect of forced expression of HOXA1 in mammary carcinoma cells, we utilized a novel Bcl-2 inhibitor that antagonizes Bcl-2 function by blocking the binding of the Bak BH3 peptide to Bcl-2 in vitro (43). Bcl-2 expression was not altered by cellular treatment with the Bcl-2 inhibitor (Fig. 4E). Use of the Bcl-2 inhibitor abrogated the protection from apoptotic cell death as a consequence of forced expression of HOXA1 (Fig. 4F) similar to the effect observed with Bcl-2 antisense oligonucleotides. The anti-apoptotic effects of forced expression of HOXA1 in mammary carcinoma cells are therefore mediated by increased Bcl-2 gene transcription.

**HOXA1 Expression in Mammary Carcinoma Cells Protects**

**FIG. 3.** Effect of forced expression of HOXA1 in mammary carcinoma cells on cell number, cell cycle progression, and apoptosis. MCF7-VECTOR and MCF7-HOXA1 cells were cultured in serum-free media or in serum-free media supplemented with either 50 nM hGH or 10% FBS as indicated. Total cell number (A), cell cycle progression (BrdUrd incorporation) (B), and apoptotic cell death (C) were determined in both cell lines under the indicated conditions as detailed under “Experimental Procedures.” Western blot analysis was utilized to determine the level of proteins involved in cell cycle progression (cyclinD1, p21<sub>waf1/cip1</sub>, and p27<sub>Kip1</sub>) (D) and apoptosis (Fas, Fadd, p53, Bax, Bak, Bel-xL, and Bcl-2) (E) as indicated. β-Actin was used as loading control. Results represent means ± S.D. of triplicate determinations. *, p < 0.01; **, p < 0.05.
against Doxorubicin-induced Apoptosis—As forced expression of HOXA1 in mammary carcinoma cells offered dramatic protection from apoptosis, we reasoned that increased HOXA1 expression would also result in decreased sensitivity to cell death as a result of exposure to anti-neoplastic agents. We therefore examined the effect of forced expression of HOXA1 on the apoptotic response of mammary carcinoma cells to doxorubicin. Doxorubicin decreased Bcl-2 promoter activity in MCF7-VECTOR cells and decreased the HOXA1-enhanced promoter activity in MCF7-HOXA1 cells, although Bcl-2 promoter activity in MCF7-HOXA1 cells treated with doxorubicin remained higher than in vehicle-treated MCF7-VECTOR cells (Fig. 5A). The level of Bcl-2 mRNA as determined by RT-PCR demonstrated concordance with Bcl-2 P1 promoter activity (Fig. 5B). Furthermore, Western blot analysis for Bcl-2 demonstrated an equivalent decrease in Bcl-2 protein upon treatment of MCF7-VECTOR cells and relative maintenance of Bcl-2 protein in doxorubicin-treated MCF7-HOXA1 cells. Bcl-xL protein was also slightly decreased after doxorubicin treatment but to equivalent levels in both cell lines. The level of Bak and Bax did not differ between the two cell lines, and no effect of doxorubicin was observed. A dramatic increase in p53 in both MCF7-VECTOR and MCF7-HOXA1 demonstrated the efficacy of doxorubicin treatment (Fig. 5C). Doxorubicin dramatically increased apoptosis in MCF7-VECTOR cells, and in comparison MCF7-HOXA1 exhibited marked resistance to the apoptotic effects of doxorubicin (Fig. 5D). Therefore, increased expression of HOXA1 in human mammary carcinoma cells produces a chemoresistant phenotype.

Forced Expression of HOXA1 in Human Mammary Carcinoma Cells Results in Increased Anchorage-independent Growth in a Bcl-2-dependent Manner—Anchorage-independent growth is one pivotal characteristic of malignant transformation (2). To determine if forced expression of HOXA1 would alter the oncogenicity of mammary carcinoma cells, we examined the anchorage-independent growth of MCF7-VECTOR and MCF7-HOXA1 in both soft agar and suspension culture. MCF7-VECTOR cells formed colonies in soft agar as expected, and forced expression of HOXA1 in MCF7-HOXA1 cells dramatically enhanced colony formation in soft agar (Fig. 6A). The size of the individual colonies formed by MCF7-HOXA1 was also significantly larger than the small cell aggregates observed with MCF7-VECTOR cells (Fig. 6D). MCF7-HOXA1 cells also increased in number significantly greater than MCF7-VECTOR cells in suspension culture and after 10 days were approximately tripled in number compared with MCF7-VECTOR cells (Fig. 6B). To determine if Bcl-2 expression was required for the observed enhancement of anchorage-independent growth as a result of forced expression of
HOXA1, we examined soft agar colony formation of MCF7-VEC-TOR and MCF7-HOXA1 cells in the presence of the Bcl-2 inhibitor. As is observed in Fig. 6C, use of the Bcl-2 inhibitor completely prevented HOXA1 enhancement of soft agar colony formation by mammary carcinoma cells (Fig. 6D). Thus, forced expression of HOXA1 enhances oncogenicity of human mammary carcinoma cells.

Forced Expression of HOXA1 Results in Oncogenic Transformation of Immortalized Human Mammary Epithelial Cells—To determine if forced expression of HOXA1 would result in oncogenic transformation of human mammary epithelial cells, we utilized the immortalized human mammary epithelial cell line MCF-10A (44). When grown attached to a plastic substrate these cells display a typical epithelial morphology, do not form colonies in soft agar, and are not capable of growth in immunocompromised mice (44). To examine the potential oncogenic capacity of HOXA1, we therefore stably transfected HOXA1 cDNA in MCF-10A cells (MCF10A-HOXA1). These cells expressed higher levels of both HOXA1 mRNA (Fig. 7A) and protein (Fig. 7B) compared with vector-transfected controls (MCF10A-VECTOR). Forced expression of HOXA1 in MCF10A-HOXA1 cells also resulted in enhanced HOXA1 transcriptional activity as evidenced by reporter activity from the EphA2-r42B enhancer (Fig. 7C). Transient transfection of the cDNA for HOXA1 binding partner PBX1 further dramatically enhanced HOXA1 transcriptional activity in MCF10A-HOXA1 cells as expected (Fig. 7C).

We therefore proceeded to examine the growth characteristics of both MCF10A-VECTOR and MCF10A-HOXA1 cells. MCF10A-HOXA1 total cell number was increased significantly greater under all examined conditions (serum free, hGH, and FBS) than MCF10A-VECTOR cell number (Fig. 7D). Comparison of nuclear BrdUrd incorporation between the two cell lines under serum-free conditions, 50 nM exogenous hGH, or FBS demonstrated that forced expression of HOXA1 significantly increased cell cycle progression of mammary carcinoma cells (Fig. 7E). Apoptotic cell death was also dramatically reduced in MCF10A-HOXA1 cells compared with MCF10A-VECTOR cells in serum-free conditions (Fig. 7F) as previously described for forced expression of HOXA1 in MCF-7 cells. Exogenous hGH slightly reduced apoptotic cell death in both MCF10A-VECTOR and MCF10A-HOXA1 cells but proportionate to the serum-free condition for each cell. FBS functioned as a powerful survival stimulus for both cell lines, although apoptosis was still less in MCF10A-HOXA1 cells compared with MCF10A-VECTOR cells (Fig. 7F).

MCF10A-HOXA1 cells formed large numerous colonies in soft agar, whereas MCF10A-VECTOR cells were largely ineffective in colonization of soft agar (Fig. 8, A and B). Thus forced expression of HOXA1 conferred tumorigenic potential upon human mammary epithelial cells. We also verified this result with a second independently generated pool of HOXA1 cDNA-transfected cell clones (data not shown). MCF10A-HOXA1 colonization of soft agar was also Bcl-2-dependent as it was entirely prevented by the Bcl-2 inhibitor described above (Fig. 8A). We also observed that forced expression of HOXA1 in

**FIG. 5.** Forced expression of HOXA1 in mammary carcinoma cells protects against doxorubicin-induced apoptosis. Forced expression of HOXA1 in mammary carcinoma cells (MCF7-HOXA1) maintains Bcl-2 promoter activity (A) and Bcl-2 mrNA levels (B) above that observed in vector-transfected control cells upon treatment of both cell lines with 1 µg/ml doxorubicin. Western blot analysis (C) demonstrates dramatic doxorubicin-induced expression of p53 in both MCF7-VECTOR and MCF7-HOXA1 cells and maintenance of Bcl-2 expression in MCF7-HOXA1 cells compared with MCF7-VECTOR cells. β-Actin was used as loading control. Doxorubicin-induced apoptotic cell death (D) was abrogated by forced expression of HOXA1 in mammary carcinoma cells as indicated. All experiments were performed as described under “Experimental Procedures.”*, p < 0.01.
mouse NIH-3T3 cells resulted in their oncogenic transformation as indicated by dramatic colony formation in soft agar in comparison to an effective lack of colony formation by vector transfected NIH-3T3 cells (data not shown).

In vitro analyses of oncogenic transformation are not always concordant with tumorigenic potential in vivo. We therefore implanted both MCF10A-VECTOR and MCF10A-HOXA1 cells into the first mammary (axillary) fat pad of female severe combined immunodeficient (SCID) mice with use of either PBS or Matrigel as vehicle. MCF10A-HOXA1 cells formed large palpable and visible tumors in all mice injected, whereas MCF10A-VECTOR cells did not (Fig. 8, C and D). The average volume of the tumors formed by MCF10A-HOXA1 cells was related to the injection vehicle with Matrigel resulting in larger tumor volume (Table I). Necropsy revealed that the tumors were attached to the underlying axillary muscle and surrounded by a vascular fibrous capsule. Histologically the neoplastic cells were locally invasive and associated with fibrous connective tissue (Fig. 8, E and F). The cells exhibited moderate cytoplasmic and nuclear pleomorphism and formed a solid mass often with areas of central necrosis. Necropsy of SCID mice injected with MCF10A-VECTOR cells failed to identify any growth. Macroscopic and histological examination of lung and liver of SCID mice injected with MCF10A-HOXA1 cells failed to identify metastatic extension of the implanted MCF10A-HOXA1 cells. Mammary carcinoma cells derived from the in vivo growth of MCF10A-HOXA1 cells expressed HOXA1 at the same level as the injected cell indicative of phenotypic retention (data not shown).

DISCUSSION

We have demonstrated here that forced expression of HOXA1 in immortalized human mammary epithelial cells (MCF-10A) results in oncogenic transformation and the development of a rapidly growing carcinoma in vivo. This is remarkable, given that forced expression of other oncogenes (e.g. Ras, Her2/neu, and TC21) are insufficient to convey tumorigenic potential on MCF-10A cells (45, 46). Furthermore, cyclin D1 (overexpressed in over 50% of human mammary carcinomas (47)), although able to support anchorage-independent growth of MCF-10A cells, does not result in tumor formation in vivo (48). However, one gene (EphA2) directly regulated by HOXA1 has also been reported to transform MCF-10A cells with consequent in vivo tumor formation (49). Indeed, here we utilized the HOX binding region of the EphA2 promoter (EphA2-r42B) to measure HOXA1 transactivation, and therefore, EphA2 may also constitute a pivotal component of the oncogenic program of HOXA1. The aberrant expression of homeobox-containing genes has been reported in a variety of neoplasias (for review see Ref. 27). However, it is debated whether homeobox-containing genes should be classified as oncogene/tumor suppressor genes per se or only as "tumor modulators" that tip the balance of tumor progression (27, 28). Herein, we provide the first direct evidence that a homeobox-containing protein is a bona fide mammary gland oncogene, the enhanced expression of which is sufficient for tumorigenesis of human mammary epithelial cells.

Primary rodent cells are easily transformed by two concomitantly introduced oncogenes (50, 51). Only recently have hu-
man cells been transformed by a combination of the genomic version of the SV40 Large T antigen, the hTERT gene that encodes the telomerase catalytic subunit, and an oncogenic allele of the H-Ras gene, H-RasV12 (52). hTERT allows mammary epithelial cells to bypass both senescence (M1) and crisis (M2) with resultant immortalization of cells. Immortalization of cells is not sufficient to create an oncogenically transformed cell (52, 53), and we have driven the oncogenic process here in immortalized human mammary epithelial cells by introduction of only the HOXA1 cDNA. Other members of the homeobox gene family have also been reported to be oncogenic (54, 55). Such examples include HOXB7 (56) and Cdx1 (57). Interestingly, certain homeobox family members have also been demonstrated to result in cellular immortalization (58). This raises the intriguing possibility that HOXA1 may both immortalize human mammary epithelial cells and result in their oncogenic transformation (shown here). Indeed, in preliminary experiments2 we have demonstrated that forced expression of HOXA1 does indeed increase transcription of the hTERT gene with a resultant increase in telomerase activity in primary human mammary epithelial cells. HOXA1 may therefore both immortalize and oncogenically transform human mammary epithelial cells. This exciting possibility is currently under active investigation in our laboratory. It is likely that autocrine production of hGH also transcriptionally regulates other homeodomain-containing proteins, and we are currently characterizing the role of two other homeobox family members in the effect of autocrine hGH on mammary carcinoma cell function.

We have observed here that HOXA1 gene expression and transcriptional activity is regulated by hGH. Since we demonstrated that forced expression of HOXA1 resulted in oncogenic transformation of human mammary epithelial cells, it raises the possibility that hGH itself is oncogenic. However, exogenous hGH does not support the anchorage-independent growth of immortalized human mammary epithelial cells3 despite the requirement of endocrine GH for optimal growth of mammary tumor xenografts (59). This is concordant with the fact that, although exogenous hGH increased HOXA1 mRNA, it did not result in an increase in HOXA1 protein nor transcriptional activity. Interestingly, however, and consistent with our previous studies (18), autocrine-produced hGH does indeed confer immortalized mammary epithelial cells with the capacity for anchorage-independent growth and tumor formation in vivo.4 Autocrine hGH may therefore utilize HOXA1 to execute its oncogenic program, and this possibility is currently under investigation. The observation that exogenous hGH regulates HOXA1 mRNA but not protein nor transcriptional activity is

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2 Y. Chen, T. Zhu, X. Zhang, and P. E. Lobie, unpublished data.

3 T. Zhu and P. E. Lobie, unpublished data.

4 T. Zhu, H. C. Mertani, X. Zhang, K.-O. Lee, and P. E. Lobie, manuscript in preparation.
suggestive that autocrine hGH production in human mammary epithelial cells also regulates HOXA1 at the translational or post-translational level. Such potential regulation of HOXA1 other than at the transcriptional level may be requisite for its transforming activity.

By use of a functional Bcl-2 inhibitor, we have demonstrated that HOXA1-dependent transformation is dependent on the interaction between Bcl-2 and Bak BH3 peptide (43). It is unlikely that this pathway constitutes the sole mechanism by which HOXA1 confers oncogenic transformation of human mammary epithelial cells and further pathways presumably will be delineated by microarray and promoter mining experiments in progress. It may be that Bcl-2 is simply required for human mammary epithelial cell survival, and functional inhibition of Bcl-2 would consequently drive the cell to apoptosis. Indeed, high concentrations of the Bcl-2 inhibitor prevented soft agar colony formation by both MCF7-VECTOR and MCF7-HOXA1 cells (data not shown). However, titration of the Bcl-2 inhibitor allowed selective inhibition of the enhanced colony formation as a consequence of forced expression of HOXA1 with no alteration in colony formation by MCF7-VECTOR cells. Thus, it is apparent that Bcl-2 is required specifically for HOXA1 enhancement of mammary carcinoma cell colony formation in soft agar.

Chemotherapeutic agents such as doxorubicin result in the induction of p53 to execute an apoptotic program (60, 61). Bcl-2 functions to prevent mitochondrial cytochrome c release and subsequent cell death (62, 63), and therefore, increased Bcl-2 expression could be expected to result in chemoresistance (64). Indeed, Bcl-2 has been observed to be overexpressed in a large percentage of human neoplasias (64), and Bcl-2 expression is associated with resistance to chemotherapy in many cancers, including mammary carcinoma (65, 67). As could be expected, forced expression of HOXA1 resulted in resistance to doxorubicin-induced cell death as a consequence of increased Bcl-2 expression. Inhibition of HOXA1 transactivation may therefore constitute a novel therapeutic target to abrogate resistance to chemotherapeutic agents. However, Bcl-2 is not the only mechanism utilized by hGH to prevent apoptotic cell death of mammary carcinoma cells, and simple antagonism of hGH signal

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TABLE I

5 × 10^6 exponentially growing MCF10A-HOXA1 and vector-transfected control cells were implanted in the presence or absence of Matrigel into the mammary fat pad of SCID mice, and tumors were harvested 3 weeks after inoculation.

| Cell               | Incidence of tumorigenicity | Mean tumor volume |
|--------------------|-----------------------------|------------------|
|                    | PBS Matrigel                | PBS Matrigel     |
| MCF10A-VECTOR      | 0/10                        | 0/10             |
| MCF10A-HOXA1       | 9/10                        | 10/10            |

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**Fig. 8.** Forced expression of HOXA1 in immortalized mammary epithelial cells results in oncogenic transformation and tumor formation in vivo. Forced expression of HOXA1 in immortalized mammary epithelial cells permits soft agar colony formation (A and B). Assays were performed as described under “Experimental Procedures.” Bcl-2 inhibitor (0.5 μg/ml) was utilized to demonstrate Bcl-2 dependence of oncogenic transformation (A). MCF10A-HOXA1 cells implanted into the first mammary (axillary) fat pad of female severe combined immunodeficient mice formed a large visible tumor mass (C and D). Histological appearance of the tumor visualized with hematoxylin and eosin (E), and local invasion of MCF10A-HOXA1 tumor cells into surrounding skeletal muscle (F). *, p < 0.01.
transduction may represent a more efficacious approach to enhance the response of mammary carcinoma cells to chemotherapy. We have previously described that autocrine hGH production promotes mammary carcinoma cell survival by at least two other mechanisms (23, 36). First, autocrine hGH increases transcription of the CHOP gene, and increased p53 mitogen-activated protein kinase-dependent CHOP-mediated transduction results in mammary carcinoma cell survival (23). Autocrine hGH production in mammary carcinoma cells also conversely results in transcriptional repression of the p53-regulated placentallike growth factor-β gene with subsequent decreases in its protein product, Smad-mediated transcription, and its cellular effects, which include cell cycle arrest and apoptosis (36). Several avenues exist for antagonism of the somatotropic axis. Non-dimerizing HGH receptor antagonists have been used clinically to treat acromegaly (68) and constitute one potential therapeutic approach. Indeed, we have utilized this antagonist in vitro to demonstrate that the effects of autocrine HGH on mammary carcinoma cell proliferation (both mitogenesis and apoptosis) are mediated via the HGH receptor (21). GH-releasing hormone antagonists have also been utilized to decrease mammary GH production and, consequently, decrease the growth of mouse mammary carcinoma xenografts (69). It is therefore conceivable that antagonism of HGH, utilized as adjuvant therapy, will increase the efficacy of conventional chemotherapeutic regimes currently utilized to treat mammary carcinoma.

In conclusion, we have demonstrated that autocrine HGH protects the expression and activity of HOXA1 in human mammary carcinoma cells. We observed that forced expression of HOXA1 protected human mammary carcinoma cells from apoptotic cell death in a Bcl-2-dependent manner. We also identified HOXA1 as a powerful oncogene for human mammary epithelial cells. Further functional characterization of HOXA1 and other hGH-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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