

HOXA1 Is Required for E-cadherin-dependent Anchorage-independent Survival of Human Mammary Carcinoma Cells*

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Forced expression of HOXA1 is sufficient to stimulate oncogenic transformation of immortalized human mammary epithelial cells and subsequent tumor formation. We report here that the expression and transcriptional activity of HOXA1 are increased in mammary carcinoma cells at full confluence. This confluence-dependent expression of HOXA1 was abrogated by incubation of cells with EGTA to produce loss of intercellular contact and rescued by extracellular addition of Ca²⁺. Increased HOXA1 expression at full confluence was prevented by an E-cadherin function-blocking antibody and attachment of non-confluent cells to a substrate by homophilic ligation of E-cadherin increased HOXA1 expression. E-cadherin-directed signaling increased HOXA1 expression through Rac1. Increased HOXA1 expression consequent to E-cadherin-activated signaling decreased apoptotic cell death and was required for E-cadherin-dependent anchorage-independent proliferation of human mammary carcinoma cells. HOXA1 is therefore a downstream effector of E-cadherin-directed signaling required for anchorage-independent proliferation of mammary carcinoma cells.

The growth and differentiation of different cell populations during development are achieved by the coordinated action of complex genetic programs. Alteration in such regulatory pathways results in oncogenic transformation and development of neoplastic lesions. Homeotic selector or homeobox (HOX)² genes are regulatory genes, which had been originally identified in *Drosophila* and encode transcription factors capable of controlling parallel developmental pathways along the antero-posterior axis of the embryo (1, 2). They exert genetic control by activating or repressing the localized expression of downstream effector genes. The effector genes of HOX genes include signaling molecules and transcription factors such as sonic hedgehog (3), fibroblast growth factors (4), bone morphogenetic proteins (5) and polycomb proteins (6) indicative that HOX genes govern cascades of regulatory pathways. HOX genes possess a homeobox, which is a 183-bp DNA sequence that encodes a 61-amino acid sequence termed as the homeodomain usually

situated at the terminal or subterminal position of the corresponding homeoprotein (7). All HOX genes bind DNA via this homeodomain, which is a helix-turn-helix DNA binding domain, and many of them have indistinguishable *in vitro* DNA recognition properties. In humans, there are 39 HOX genes present in four paralogous gene clusters named as the HOX-A, HOX-B, HOX-C, and HOX-D, which had been suggested to have resulted by duplication during the course of evolution. The HOX genes are arranged in a linear order and are shown to be responsible for patterning the body by control of cell identity, cell growth, cell differentiation (8), and cell-cell and cell-extracellular matrix interactions (9). Recently altered expression of HOX genes have also been linked to a variety of human cancers, including those of kidney (including Wilms' tumor), colon, prostate, skin, and small-cell lung carcinoma (10–13).

In the mammary gland HOX genes are expressed in a distinct pattern during development. For example HOXC6 is expressed during puberty and at maturity but is not expressed during pregnancy due to its regulation by steroid hormones (14). It has also been demonstrated that the expression level of certain HOX genes such as HOXA5 and HOXC6 are repressed in mammary adenocarcinoma (13). HOXA5 has been demonstrated to stimulate mammary carcinoma cell apoptosis by both p53-dependent and -independent mechanisms (13, 16). In contrast, HOXA1 is not expressed in the differentiated mammary gland but is up-regulated in neoplastic lesions of the human mammary gland (17, 18). Recently, we have demonstrated that autocrine human growth hormone, a major regulator of human mammary gland development and a potential human mammary epithelial oncogene (19), up-regulated HOXA1 in the human mammary epithelial cell (20, 21). HOXA1 stimulates the transcriptional activation of a number of pro-oncogenic molecules, including cyclinD1 and *Bcl-2*, thereby permitting increased proliferation and survival of human mammary carcinoma cells (21). HOXA1 also governs the transcriptional program stimulated by autocrine human growth hormone required for autocrine human growth hormone-stimulated oncogenic transformation of human mammary epithelial cells, including transcriptional up-regulation of *c-Myc*, cyclin D1, and *Bcl-2* (19). Forced expression of HOXA1 is sufficient to oncogenically transform immortalized human mammary epithelial cells, with consequent soft agar colony formation and aggressive *in vivo* tumor formation (21).

Survival of normal epithelial cells depends on signals generated by the interaction of cells with the basement membrane, and in the absence of anchorage the cells exhibit the molecular characteristics of apoptosis and are deleted (22, 23). This form of apoptosis is called anoikis, and it is believed to preclude epithelial cells from survival, reattachment, and proliferation outside the appropriate tissue context (24, 25). In contrast, many carcinoma cells are anoikis-resistant and survive in the absence of

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² The abbreviations used are: HOX, homeobox selector; RT, reverse transcription; PBS, phosphate-buffered saline; BrdUrd, bromodeoxyuridine; TRITC, tetramethylrhodamine isothiocyanate; HBSS, Hanks' balance salt solution; MEK1, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1; PLL, poly-L-lysine; EMT, epitheliomesenchymal transition; MAP, mitogen-activated protein.

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contact with the basement membrane, inside three-dimensional tumor nests and in the absence of matrix attachment during metastasis (25). Some of the mechanisms that confer anoikis resistance on epithelial cells during tumor progression have been described (25, 26). Previous work has implicated E-cadherin-mediated cell-cell adhesion in protecting specific epithelium-derived cells from anoikis (27, 28). In addition to mediation of intercellular adhesion, E-cadherin also stimulates intracellular signaling (29) and has been demonstrated to potentiate cell survival. It has been demonstrated that E-cadherin suppresses apoptosis of immortalized granulosa cells (30) and that experimental truncation of E-cadherin results in potentiation of apoptotic cell death in prostate epithelial cells (31). N-cadherin can also mediate cell survival in several cell types (32–34). In addition, E-cadherin has been demonstrated to stimulate anchorage-independent growth and survival of squamous carcinoma cells (27).

During the course of our studies on the regulation of HOXA1 in human mammary carcinoma cells (21), we noted that the expression and consequent transcriptional activity of HOXA1 was confluence-dependent, suggestive of possible E-cadherin-dependent regulation of HOXA1. Herein, we demonstrate that E-cadherin-directed signaling through Rac1 up-regulates HOXA1 in human mammary carcinoma cells. Increased HOXA1 expression stimulated by E-cadherin-activated signaling decreased apoptotic cell death and allowed anchorage-independent proliferation in human mammary carcinoma cells. Thus, E-cadherin-mediated intercellular adhesion utilizes HOXA1 to suppress apoptosis and promote anchorage-independent growth.

EXPERIMENTAL PROCEDURES

Cell Culture—The MCF-7 and MCF-10A cell lines were obtained from the ATCC. MCF-7 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine. MCF-10A cells were cultured in Dulbecco's modified Eagle's medium/F-12 medium (Invitrogen) supplemented with 5% horse serum (Invitrogen) plus 2 mM glutamine, 100 μ g/ml streptomycin, 100 IU/ml penicillin, 0.25 μ g/ml ampicillin B, 100 ng/ml cholera toxin, 20 ng/ml epidermal growth factor (Upstate Biotechnology, Lake Placid, NY), 0.5 μ g/ml hydrocortisone (Calbiochem), and 10 μ g/ml insulin. The MCF-7 cell line was stably transfected with a HOXA1 expression plasmid (pSG5-HOXA1) and pcDNA3 empty vector in a ratio of 5:1 by use of Effectene transfection reagent obtained from Qiagen as described previously (21). pSG5-HOXA1 is the generous gift of Dr. Vincenzo Zappavigna (Milano, Italy). Positive transfectants were selected in 800 μ g/ml G418 (Calbiochem). Individual colonies were selected to determine the HOXA1 expression level. The cell line MCF7-HOXA1 was established by pooling five individual colonies exhibiting forced expression of HOXA1. Likewise, MCF-7 cells, stably transfected with vector, were also established (MCF7-VECTOR) as control.

Reverse Transcriptase-PCR—Extraction of total RNA and RT-PCR were performed as described previously (20). To compare the PCR products semi-quantitatively, 15–40 cycles of PCR (annealing temperature, 55 °C) were performed to determine the 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'-GGGAAAGTTGGAGAGTACGGC-3'; HOXA1 (antisense), 5'-CCTCAGTGGGAGGTAGTCAG-3'; β -actin (sense), 5'-ATGATATCGCCGCGCTCG-3'; and β -actin (antisense), 5'-CGCTCGGTGAGGATCTTCA-3'.

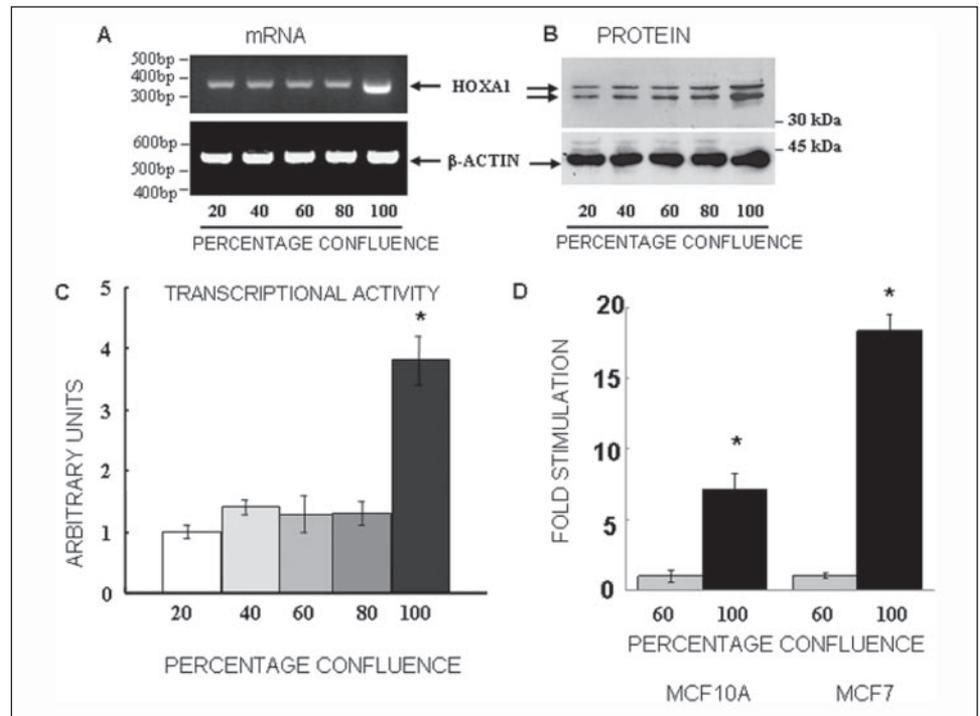
Luciferase Reporter Assay for EphA2-r42B and Bcl-2 Promoter Constructs—Cells were cultured in six-well plates. Transient transfection was performed by use of Effectene as described (20). Briefly, 0.2 μ g of the respective luciferase constructs (and other constructs as appropriate) were transfected per well in serum-free RPMI medium for 12 h before the medium was changed to fresh serum-free RPMI. 0.2 μ g of β -galactosidase reporter vector was co-transfected as the control for transfection efficiency. After a further 24 h, cells were washed with PBS, and luciferase assays were performed as described previously (21). β -Galactosidase activity was measured in the assay buffer (100 mM sodium phosphate, pH 7.3, 1 mM MgCl₂, 50 mM β -mercaptoethanol, 0.665 mg/ml *o*-nitrophenyl- β -D-galactopyranoside). Results were normalized to the level of β -galactosidase activity and protein concentration in the samples. *EphA2-r42B-LUC* was a generous gift of Dr. Jin Chen (Nashville, TN). The *Bcl-2* P1 promoter reporter plasmid was a kind gift of Dr. John Kurland (Houston, TX).

Real-time PCR—Total RNA was isolated as described above, and the first strand cDNA synthesis with oligo(dT) primers was performed using a Reverse Transcription Kit (Applied Biosystems, Newark, NJ) according to the manufacturer's instructions. Real-time PCR for HOXA1 (Inventoried Assay ID Hs00171793-m1, Applied Biosystems) and glyceraldehyde-3-phosphate dehydrogenase (Inventoried Assay ID Hs99999905-m1, Applied Biosystems) were performed in triplicate on 1.00 μ g of cDNA using Taq Man PCR Master Mix in a reaction volume of 25 μ l. PCR amplification and detection was performed using an ABI Prism 7900 HT (Applied Biosystems). Exponential amplification of all PCR reactions ranged from 1.95 to 2.00 across seven serial log dilutions of template. Amplification of a single product for each primer set was confirmed by dissociation curve analysis, and all products were visualized after electrophoresis on 2% agarose gel. The comparative C_t method for relative quantification ($2^{-\Delta\Delta C_t}$) was used to quantitative gene expression according to the recommendations of Applied Biosystems (7900 HT Real-Time fast and SDS enterprise and data base user guide). Expression of target genes was normalized to the level of glyceraldehyde-3-phosphate dehydrogenase, and the -fold difference was calculated.

Western Blot Analysis—Cells were treated and harvested as described. Whole cell lysates were prepared according to the protocol described (20, 21) and normalized for protein content. Proteins were resolved by SDS-polyacrylamide (12%) gel electrophoresis, transferred to a nitrocellulose membrane, and blotted with the antibodies as indicated. The anti- β -actin, anti-HOXA1, anti-E-cadherin, anti- α -catenin, anti- β -catenin, and anti- γ -catenin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); immunolabeling was visualized by using an ECL detection kit from Amersham Biosciences according to the manufacturer's instruction.

Confocal Laser Scanning Microscopy—MCF-7 and MCF10-A cells were grown in cavity slides to the appropriate confluence (60 or 100%), fixed in 4% paraformaldehyde/PBS, pH 7.4, washed, and blocked with BBX (PBS 0.1% Triton, 0.1% bovine serum albumin, 250 mM NaCl). The cells were incubated with anti-HOXA1 and anti-E-cadherin antibodies overnight at 4 °C. They were again washed and blocked with BBX and incubated with the anti-Goat-TRITC and anti mouse-Alexa488 secondary antibodies in BBX and mounted. Cells were cultured to the appropriate confluence (60 or 100%) and treated with the E-cadherin function-blocking antibody DECMA-1 or control IgG and processed as above. Labeled cells were visualized with a Carl Zeiss 510 Meta confocal laser system. Images were converted to the tagged-information-file format and processed with Adobe Photoshop.

FIGURE 1. HOXA1 mRNA, protein, and transcriptional activity are increased in mammary carcinoma cells (MCF-7) at full confluence. MCF-7 cells were cultured at different cellular densities (20–100% confluence). Experiments were performed as described under “Experimental Procedures.” The level of HOXA1 mRNA was determined by RT-PCR (A), HOXA1 protein by Western blot analysis (B), and HOXA1 transcriptional activity by reporter assay (C) as indicated. β -Actin was used as loading control. D, MCF-7 cells and mammary epithelial cells (MCF-10A) were cultured at subconfluence (60% confluence) and full confluence (100% confluence) and the level of HOXA1 mRNA quantified by real-time PCR. *, $p < 0.01$.



5'-Bromo-2'-deoxyuridine Incorporation Assay—Mitogenesis was directly assayed by measuring the incorporation of BrdUrd as described (20, 21). For incorporation of BrdUrd, subconfluent cells were pulse-labeled with 20 mM 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 instructions. 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).

Measurement of Apoptosis—Apoptotic cell death was measured by fluorescent microscopic analysis of cell DNA-staining patterns with Hoechst 33258 from Sigma Co. (20, 21). Cells were trypsinized with 0.5% trypsin and washed twice with serum-free medium. The cells were then seeded to glass coverslips in six-well plates and incubated in serum-free medium with or without E-cadherin expression plasmid, HOXA1 expression plasmid, DECMA-1, and HOXA1 RNA interference (19) 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 μ g/ml) for 10 min at room temperature. Following washing with PBS, nuclear morphology 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 eight random microscopic fields at $\times 400$ magnification.

Determination of Anchorage-independent Growth of Cells—Cells were trypsinized and dispensed as either single cells (non-aggregated) or in aggregated clumps by differential cycles of mechanical aspiration. Cells (5×10^4) in suspension culture were grown in 30-mm plastic bacteriological dishes (Sterilin, Teddington, UK). On the days indicated, cells were harvested and counted as for monolayers (21).

Calcium Switch—For calcium switch experiments, MCF-7 cells were seeded into dishes, cultured for 24 h, and then the cells were serum-

starved. After 24-h incubation, E-cadherin-mediated cell-cell contacts were disrupted by treatment with 4 mM EGTA at 37 $^{\circ}$ C for 30 min. Thereafter, intercellular contacts were allowed to reform in the presence of normal Ca^{2+} containing medium ($\text{CaCl}_2 \sim 1.8$ mM) at 37 $^{\circ}$ C for various time points with or without E-cadherin function-blocking antibodies (DECMA clone-1 at 1:100 dilution, Sigma-Aldrich).

E-cadherin Cell Adhesion Assays (hE/Fc Experiments)—The recombinant protein (hE/Fc) consisting of the complete ectodomain of human E-cadherin fused to the Fc region of IgG was produced as described (35). Substrata adsorbed with hE/Fc supported E-cadherin-specific adhesion and contact formation (36). Using this model, E-cadherin engagement is induced without cell-cell contact (36). For hE/Fc experiments, bacterial 10-cm dishes (precoated with nitrocellulose) were coated with hE/Fc (50 μ g/ml) for 2 h at room temperature. Blocking of nonspecific binding sites was performed with 1% bovine serum albumin in Hanks' balanced salt solution (HBSS) supplemented with 5 mM CaCl_2 for 1 h and 30 min at room temperature. Dishes were washed five times before addition of cells. MCF-7 cells were washed once with 10 mM EDTA in HBSS/ CaCl_2 for 1 min and directly trypsinized in 0.01% crystalline trypsin in HBSS/ CaCl_2 for 15 min at 37 $^{\circ}$ C. MCF-7 cells were then washed with HBSS/ CaCl_2 and resuspended in HBSS/ CaCl_2 supplemented with 0.05% fetal calf serum, seeded onto the 10-cm dishes and incubated at 37 $^{\circ}$ C. Thirty minutes after seeding, non-adhered cells were carefully removed by exchange of media. Five hours after seeding the cells onto the dishes, RNA and protein extraction were performed.

Statistics—All experiments were repeated at least three to five times. All numerical data are expressed as mean \pm S.D. Data were analyzed using the two-tailed *t* test or analysis of variance.

RESULTS

HOXA1 mRNA, Protein, and Transcriptional Activity Are Increased in Mammary Carcinoma Cells at Full Confluence—The level of HOXA1 mRNA in mammary carcinoma cells MCF-7 at varying degrees of confluence and at full confluence was first examined by semi-quantitative RT-PCR. Fig. 1A demonstrated an amplified fragment of the

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predicted size (359 bp) appropriate for HOXA1 mRNA in MCF-7 cells (21). HOXA1 mRNA was increased in MCF-7 cells at full confluence (100% confluence) in comparison to subconfluent conditions (20%, 40%, 60%, and 80% confluences). The level of β -actin mRNA did not differ in MCF-7 cells at different degrees of confluency and was used as

a control for RNA quality (Fig. 1A). To determine if the increased HOXA1 mRNA observed in MCF-7 cells at full confluence resulted in increased HOXA1 protein, we examined the level of HOXA1 protein in MCF-7 cells at the corresponding varying degrees of confluence by Western blot analysis. Both the 33-kDa and the 35-kDa forms of HOXA1 were detectable as described previously (21) (Fig. 1B) with increased HOXA1 protein observed at 100% confluence. We next determined if HOXA1-mediated transcription was consequently increased in MCF-7 cells at full confluence, by luciferase reporter activity from the EphA2-r42B enhancer, which contains four HOX-PBX binding sites (37). MCF-7 cells at full confluence (100% confluence) exhibited increased luciferase activity from the EphA2-r42B enhancer compared with MCF-7 cells at subconfluence (20%, 40%, 60%, and 80% confluences) (Fig. 1C), indicative of increased HOXA1 transcriptional activity.

To verify the effect of confluence on HOXA1 mRNA expression in mammary epithelial derived cell lines we utilized quantitative real-time PCR analysis to determine the expression of HOXA1 at subconfluence (60%) and confluence (100%) in both MCF-7 cells and a mammary epithelial (MCF-10A) cell line. In this instance, the expression of HOXA1 was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase. The relative expression of HOXA1 mRNA was increased 19- and 7-fold in MCF-7 and MCF-10A cells, respectively, at confluence compared with subconfluence (Fig. 1D). Thus, confluent MCF-7 cells exhibited elevated HOXA1-mediated transcriptional activity compared with subconfluent cultures.

The Increase in HOXA1 Expression in Mammary Carcinoma Cells at Full Confluence Is Cell-Cell Adhesion-dependent—The elevation in HOXA1 expression in confluent cultures of MCF-7 cells prompted us to examine the role of E-cadherin-mediated cell-cell attachment in the increase in HOXA1 expression that occurs at confluence. We first used EGTA addition to media to chelate Ca^{2+} , thereby disrupting E-cadherin-mediated cell-cell contact (38). As shown in Fig. 2A, use of EGTA completely abrogated the increased expression of HOXA1 mRNA observed at full confluence in MCF-7 cells. Similarly, analysis of HOXA1 transcriptional activity by use of the EphA2-r42B enhancer demonstrated abrogation of increased HOXA1-mediated transcriptional activity in MCF-7 cells at full confluence by EGTA (Fig. 2B). Luciferase activity generated by a constitutively active luciferase vector (pSV2-LUC) was not altered in the presence of EGTA (Fig. 2C) indicative that EGTA did not interfere with transcription or translation of the luciferase reporter.

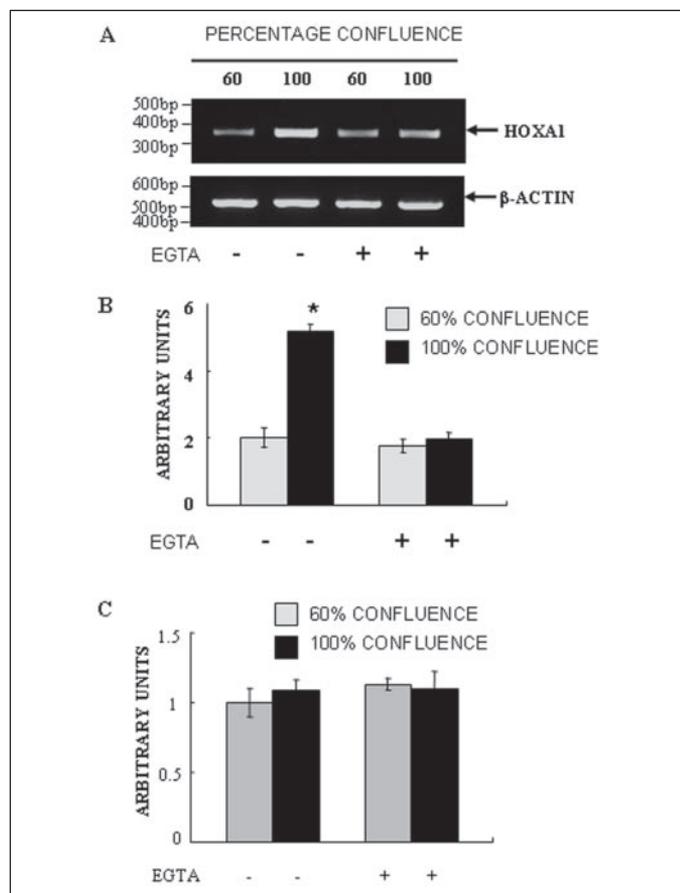


FIGURE 2. The increase in HOXA1 expression in mammary carcinoma cells at full confluence is cell-cell adhesion dependent. MCF-7 cells were cultured at subconfluence (60% confluence) and full confluence (100% confluence) in the presence and absence of EGTA to disrupt cell to cell adhesion. The level of HOXA1 mRNA was determined by RT-PCR (A) and the level of HOXA1 transcriptional activity was determined by reporter assay (B). Luciferase activity generated by a constitutively active luciferase vector (pSV2-LUC) was not altered in the presence of EGTA (C). *, $p < 0.01$.

FIGURE 3. Intercellular adhesion stimulated increase in HOXA1 is E-cadherin-dependent. Confluent MCF-7 cells were incubated with 4 mM EGTA. After 30 min, the medium was replaced with RPMI containing Ca^{2+} for the indicated time periods in the presence of a control IgG or the E-cadherin function blocking antibody DECMA-1. At the indicated times, cells were lysed and the level of HOXA1 mRNA was determined by RT-PCR (A and C) and HOXA1 protein by Western blot analysis (B and D). β -Actin was used as loading control.

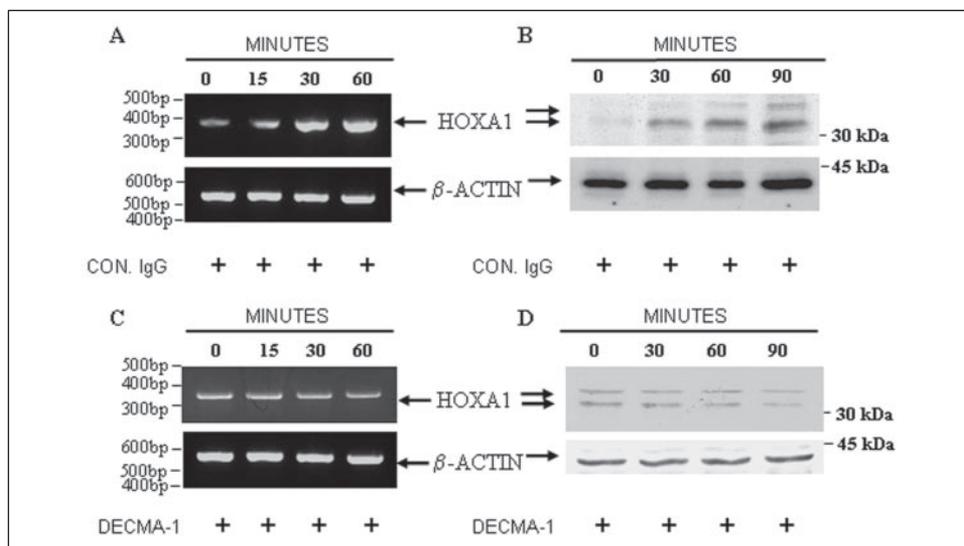
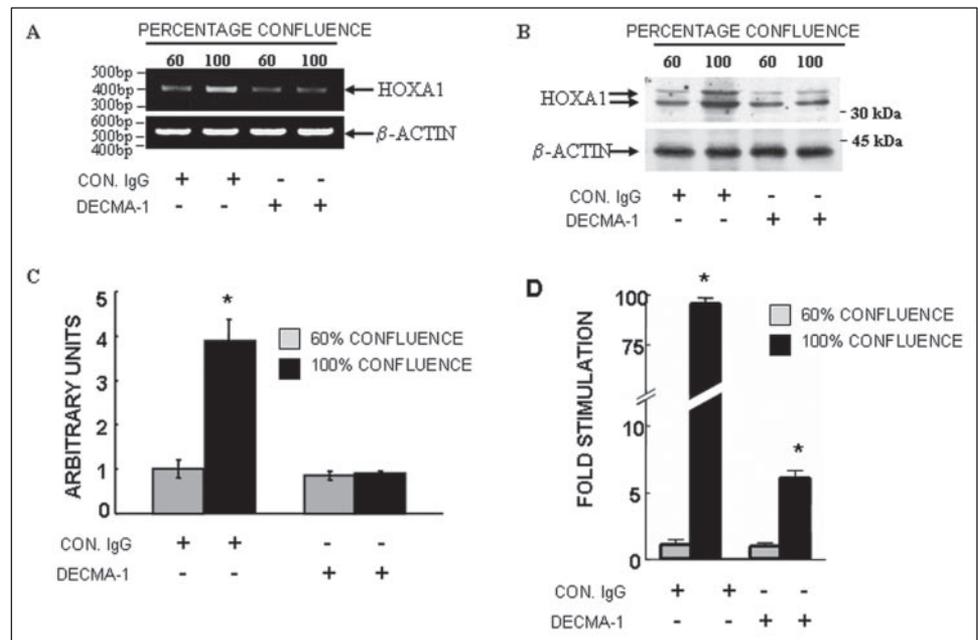


FIGURE 4. The increase in HOXA1 expression in mammary epithelial (MCF-10A) and mammary carcinoma cells (MCF-7) at full confluence is E-cadherin-dependent. MCF-7 cells were cultured at subconfluence (60% confluence) and full confluence (100% confluence) in the presence and absence of DECMA-1, a function-blocking antibody against E-cadherin. The level of HOXA1 mRNA was determined by RT-PCR (A), HOXA1 protein by Western blot analysis (B), and HOXA1 transcriptional activity by reporter assay (C) as indicated. D, MCF-10A cells were cultured at subconfluence (60% confluence) and full confluence (100% confluence) in the presence and absence of DECMA-1, and the level of HOXA1 mRNA was quantified by real-time PCR. *, $p < 0.01$.



Using the calcium switch model in MCF-7 cells (35, 38), we examined the dynamics of HOXA1 mRNA expression by time-lapse analysis (Fig. 3A). E-cadherin-mediated cell-cell adhesion was abrogated by chelation of Ca^{2+} and then re-initiated by re-introducing Ca^{2+} (38). This approach produces a time-dependent accumulation of E-cadherin and formation of adherens junctions in MCF-7 cells (39). Restoration of E-cadherin-mediated adhesion by Ca^{2+} significantly increased the expression of HOXA1 mRNA and protein in MCF-7 cells (Fig. 3, A and B). The increase in HOXA1 mRNA and protein expression was time-dependent. Increased HOXA1 expression was detected at 15 min (HOXA1 mRNA) and 30 min (HOXA1 protein) and peaked at 60 min (HOXA1 mRNA) and 90 min (HOXA1 protein) (Fig. 3, A and B). Thus, the increase in HOXA1 expression in mammary carcinoma cells at full confluence is mediated by cell-cell adhesion.

MCF-7 cells adhere to each other through not only E-cadherin but also other adhesion molecules, and it is therefore possible that cell adhesion molecules besides E-cadherin may increase HOXA1 upon intercellular adhesion. The calcium switch method was therefore performed in the presence of DECMA-1. MCF-7 cells were incubated with EGTA, and Ca^{2+} was subsequently restored to the medium in the presence of DECMA-1, a function-blocking antibody directed against E-cadherin (40). The increase in HOXA1 mRNA and protein expression induced by calcium restoration was blocked by DECMA-1 (Fig. 3, C and D), indicative that E-cadherin is pivotal for increased HOXA1 expression at full confluence. Thus, the increase in HOXA1 expression in mammary carcinoma cells upon intercellular adhesion is E-cadherin-dependent.

The Increase in HOXA1 Expression in Mammary Carcinoma Cells at Full Confluence Is E-cadherin-dependent—To definitively determine that increased HOXA1 expression observed in mammary carcinoma cells at full confluence was due to E-cadherin, we tested the effect of DECMA-1 on the expression of HOXA1 at subconfluence and confluence. As is observed in Fig. 4 (A and B), use of DECMA-1 in MCF-7 cells completely abrogated the increased expression of HOXA1 mRNA and protein at full confluence. In addition, use of DECMA-1 also diminished the increased expression of HOXA1 mRNA at full confluence in MCF-10A cells as demonstrated by real-time PCR analysis (Fig. 4D). Furthermore, use of DECMA-1 in MCF-7 cells abrogated the increased HOXA1-mediated transcriptional activity at full confluence (Fig. 4C).

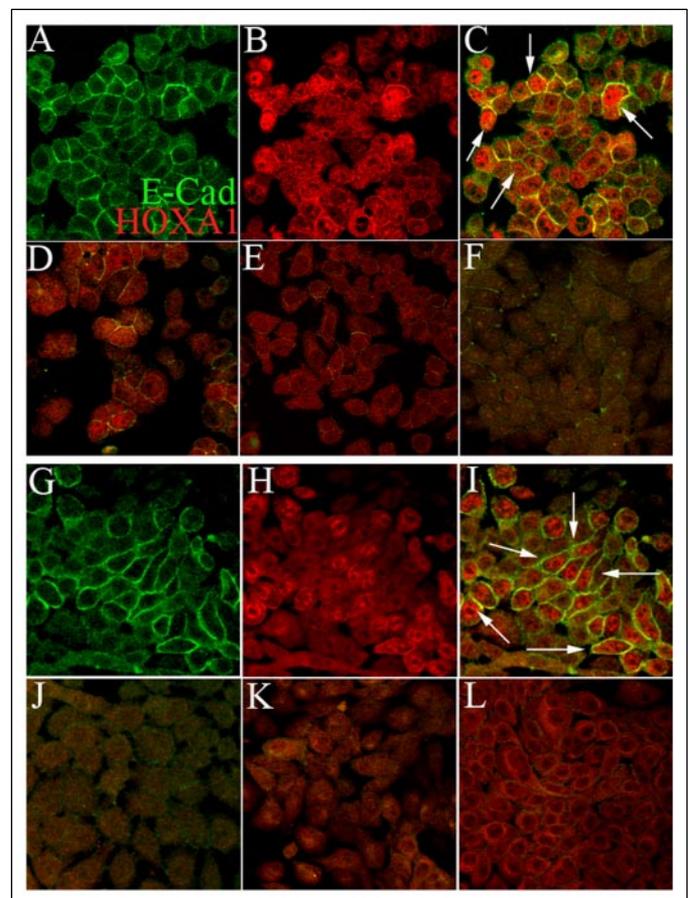
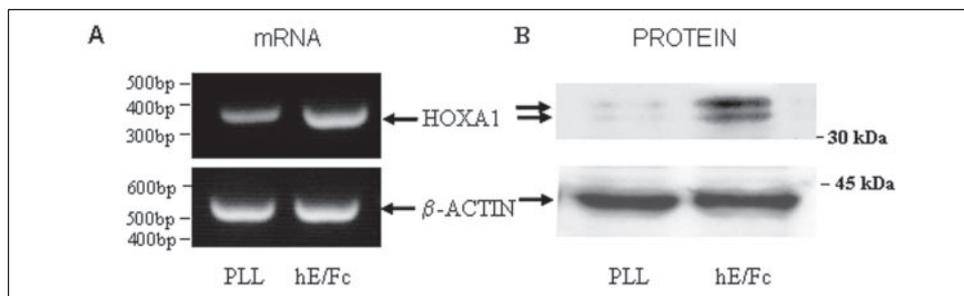


FIGURE 5. Confocal laser scanning microscopic analysis of the effect of confluence on HOXA1 expression in mammary epithelial (MCF-10A) and mammary carcinoma cells (MCF-7). MCF-7 (A–F) and MCF-10A (G–L) cells were cultured at subconfluence (60% confluence) (D, E, J, and K) and full confluence (100% confluence) (A–C, F, G–I, and L) in the presence (E, F, K, and L) and absence (A–D and G–J) of DECMA-1. E-cadherin (green) and HOXA1 (red) were localized as described under “Experimental Procedures.” Arrows indicate cells exhibiting prominent membrane localization of E-cadherin at the site of intercellular contact with increased HOXA1 expression.

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FIGURE 6. The increase in HOXA1 expression in mammary carcinoma cells at full confluence is regulated by E-cadherin-activated signaling. Subconfluent MCF-7 cells were plated onto poly-L-lysine-coated (PLL) or hE/Fc-coated substrata. The level of HOXA1 mRNA was determined by RT-PCR (A) and HOXA1 protein by Western blot analysis (B). β -Actin was used as loading control.



Luciferase activity generated by a constitutively active luciferase vector (pSV2-LUC) was not altered in the presence of DECMA-1 (data not shown) indicative that DECMA-1 did not interfere with transcription nor translation of the luciferase reporter.

We also utilized confocal laser scanning microscopy to demonstrate the effect of confluence and E-cadherin on HOXA1 expression in both MCF-7 and MCF-10A cells. At full confluence in both cell lines, E-cadherin was prominently localized to the membrane at sites of intercellular contact (Fig. 5, A–C and G–I). In these cells with prominent membrane localization of E-cadherin, we observed increased expression of HOXA1 relative to subconfluent cells without prominent localization of E-cadherin to the membrane. Some cells at subconfluence with partial intercellular contact also exhibited partial localization of E-cadherin to the membrane and increased HOXA1 expression. Use of DECMA-1 dramatically disrupted E-cadherin localization to the membrane and decreased the expression of HOXA1 to levels observed in subconfluent as well as confluent cells in both MCF-7 and MCF-10A cells (Fig. 5, E, F, K, and L).

We also examined for a potential physiological association between the expression of E-cadherin and HOXA1 by use of the Oncomine data base (www.oncomine.org). We observed a positive correlation ($r = 0.56$) between the expression of E-cadherin and HOXA1 mRNAs in samples of normal mammary gland. Expression of HOXA1 in the human mammary epithelial cell is therefore E-cadherin-dependent.

The Increase in HOXA1 Expression in Mammary Carcinoma Cells at Full Confluence Is Regulated by E-cadherin-activated Signaling—The results above do not distinguish whether the increase in HOXA1 at full confluence is a direct downstream consequence of E-cadherin ligation or simply a result of juxtacrine signals that required E-cadherin adhesion to bring cell surfaces together. We therefore next delineated whether increased HOXA1 expression resulted from direct E-cadherin-activated signals or E-cadherin-dependent juxtacrine signals. To achieve this, we utilized a recombinant protein (hE/Fc) consisting of the complete ectodomain of human E-cadherin fused to the Fc region of IgG. Substrata adsorbed with hE/Fc support E-cadherin-specific contact formation and adhesion of cells (29). Thus, using this model, E-cadherin homophilic ligation is achieved without cell-cell contact. HOXA1 mRNA was increased in MCF-7 cells adhered to hE/Fc-coated substrata in comparison to MCF-7 cells adhered to poly-L-lysine (PLL), which was used as a control to hE/Fc. The level of β -actin mRNA did not differ under the different conditions and was also used as a control for RNA quality (Fig. 6A). We further examined the level of HOXA1 protein in MCF-7 cells adhered to either hE/Fc-coated substrata or PLL. As is observed in Fig. 5B, both the 33- and the 35-kDa forms of HOXA1 were detected, with increased HOXA1 protein observed in MCF-7 cells adhered to hE/Fc-coated substrata (Fig. 6B). All these experiments were performed with MCF-7 cells at subconfluence without cell-cell contact. Thus, increased HOXA1 expression in mammary carcinoma cells

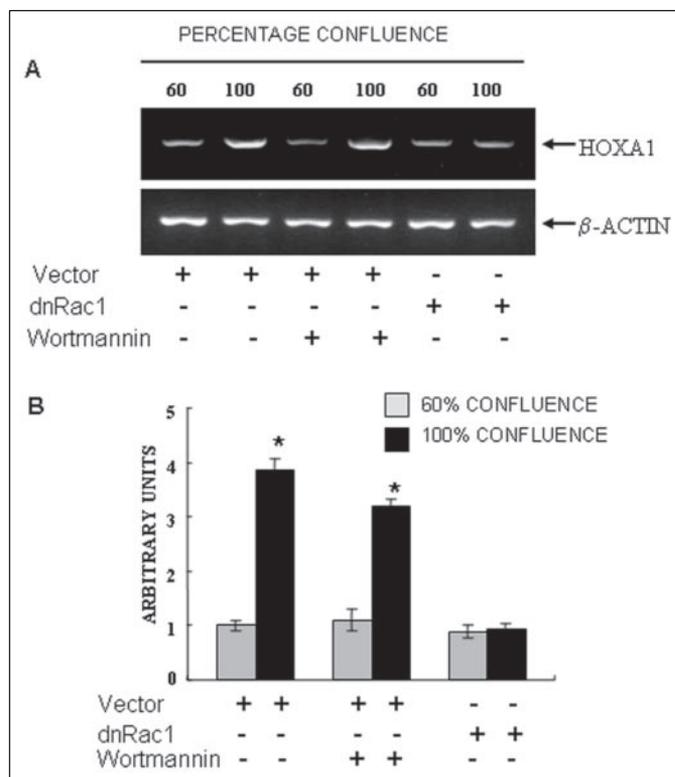


FIGURE 7. Rac1 is required for the confluence-dependent increase in HOXA1 expression in mammary carcinoma cells. MCF-7 cells were cultured at subconfluence (60% confluence) and full confluence (100% confluence) and transiently transfected with DNRac1 (dominant negative N17 Rac1 mutant) or treated with the phosphatidylinositol 3-kinase inhibitor wortmannin. The N17 Rac1 (DNRac1) mutant abrogated E-cadherin-increased HOXA1 mRNA expression (A) and HOXA1-mediated transcriptional activity at full confluence (B), whereas wortmannin (60 nM) did not (A and B). *, $p < 0.01$.

results from direct E-cadherin-activated signaling and not E-cadherin-dependent juxtacrine signaling.

Rac1 Is Specifically Required for the E-cadherin-activated Increase in HOXA1 Expression in Mammary Carcinoma Cells—The α -, β -, and γ -catenins, along with E-cadherin, are key components of the adherens junction (41). To exclude the possibility that confluence alters the expression of these molecules, we examined their protein levels in MCF-7 cells at subconfluence and confluence. The levels of E-cadherin and α -, β -, and γ -catenins did not significantly differ between the cells at subconfluence (60% confluence) and full confluence (100% confluence). Equal loading of the cell extracts was verified by reprobing the stripped membrane for β -actin (data not shown).

Phosphatidylinositol 3-kinase and Rac1 have been demonstrated to be directly activated by E-cadherin (29). To determine if phosphatidylinositol 3-kinase or Rac1 was responsible for the E-cadherin-stimulated increase in HOXA1 expression, we inhibited the activity of phosphati-

FIGURE 8. Rac1 is required for the E-cadherin-activated increase in HOXA1 expression in mammary carcinoma cells. Subconfluent MCF-7 cells transiently transfected with vector or N17Rac1 were plated onto poly-L-lysine-coated (PLL) or hE/Fc-coated substrata. N17Rac1 prevented the increased HOXA1 mRNA (A) and protein (B) observed in MCF-7 cells adhered to hE/Fc-coated substrata. β -Actin was used as loading control.

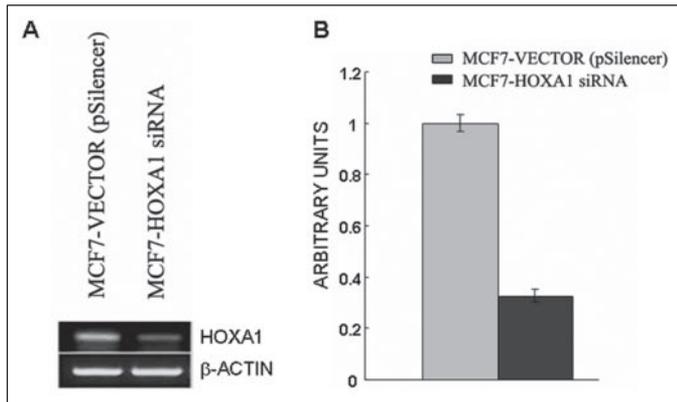
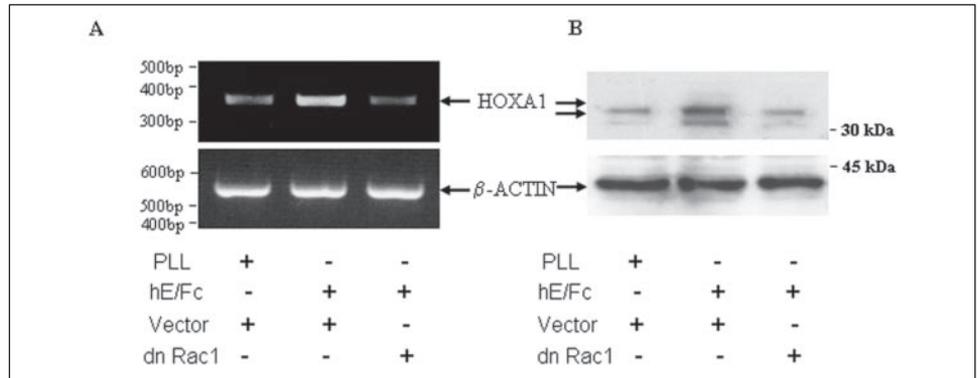


FIGURE 9. siRNA mediated depletion of HOXA1. siRNA to HOXA1 was generated as described under "Experimental Procedures." MCF-7 cells were transiently transfected with HOXA1 siRNA. A, RT-PCR was utilized to demonstrate depletion of HOXA1 mRNA by siRNA. B, siRNA abrogation of HOXA1-mediated transcriptional activity.

dylinositol 3-kinase and Rac1 in MCF-7 cells by use of wortmannin and a dominant negative N17 Rac1 mutant (42), respectively. Transient expression of the dominant negative N17 Rac1 (dnRac1) mutant significantly inhibited both the E-cadherin-increased HOXA1 mRNA expression and increased HOXA1-mediated transcriptional activity at full confluence (Fig. 7, A and B), whereas wortmannin did not significantly affect the level of HOXA1 expression (Fig. 7, A and B). To further verify the involvement of Rac1 in the direct E-cadherin-stimulated increase in HOXA1, we examined HOXA1 mRNA and protein levels in MCF-7 cells adhered to hE/Fc or PLL-coated substrata with and without forced expression dnRac1. As is observed in Fig. 8 (A and B), use of dnRac1 completely prevented the increased HOXA1 mRNA and protein expression observed in MCF-7 cells adhered to hE/Fc-coated substrata. Thus, Rac1 activity is required for the E-cadherin stimulated increase in HOXA1 expression in mammary carcinoma cells.

Increased HOXA1 Expression by E-cadherin-activated Signaling Enhances Survival of Human Mammary Carcinoma Cells—HOXA1 has previously been demonstrated to offer dramatic protection from apoptosis in MCF-7 cells as a consequence of transcriptional regulation of Bcl-2 (21). To determine the role of E-cadherin stimulated HOXA1 expression we first constructed a siRNA molecule (19) to deplete the level of HOXA1 mRNA. Targeting of HOXA1 by siRNA resulted in depleted HOXA1 mRNA within the cell and abrogated HOXA1-mediated transcriptional activity (Fig. 9). We have previously controlled for potential off-target effects of this siRNA molecule (19).

We therefore first examined whether increased HOXA1 expression consequent to E-cadherin-activated signaling would offer protection from apoptosis in MCF-7 cells. MCF-7 cells at full confluence exhibited reduced apoptosis compared with MCF-7 cells at subconfluence when

cultured in serum-free medium (Fig. 10A). Transient forced expression of E-cadherin reduced apoptotic cell death in MCF-7 cells at full confluence (Fig. 10A), whereas it did not significantly alter apoptosis at subconfluence. The E-cadherin function blocking antibody DECMA-1 completely prevented the decreased apoptotic cell death observed in MCF-7 cells at full confluence (Fig. 10B), whereas it did not significantly alter apoptosis in MCF-7 cells at subconfluence. Transient forced expression of HOXA1 significantly reduced apoptotic cell death in MCF-7 cells at both confluence and subconfluence (Fig. 10C). Depletion of HOXA1 by use of HOXA1 siRNA (19) completely abrogated increased cell survival observed at full confluence and further increased the apoptotic rate of subconfluent cells (Fig. 10D). HOXA1 siRNA also prevented the increase in cell survival observed upon forced expression of E-cadherin in confluent cells (Fig. 10E). If the E-cadherin-activated increase in HOXA1 was required for the observed decrease in apoptosis at full confluence, we reasoned that inhibition of cell survival by blocking of E-cadherin function would be rescued by forced expression of HOXA1. Transient forced expression of HOXA1 completely reversed the increase in apoptosis after DECMA-1 inhibition of E-cadherin function in confluent cells (Fig. 10F). Expression of E-cadherin and HOXA1 for these experiments was verified by Western blot analysis (data not shown). Thus, increased HOXA1 expression consequent to E-cadherin-activated signaling resulted in increased survival of human mammary carcinoma cells.

The ability of HOXA1 to stimulate cell survival has previously been demonstrated to be mediated by transcriptional activation of the Bcl-2 gene via the P1 promoter (21). Similarly, E-cadherin has also been demonstrated to regulate the expression of Bcl-2 to mediate cellular survival (28). We therefore examined the effect of cell confluence on mammary carcinoma cell transcription of the Bcl-2 gene to provide a mechanism for the above observed effects. Mammary carcinoma cells at full confluence exhibited significantly higher activation of the Bcl-2 promoter compared with cells at subconfluence (Fig. 11). Disruption of E-cadherin with DECMA-1, or depletion of HOXA1 with siRNA, both reduced the confluence dependent increase in Bcl-2 gene transcription. Furthermore, forced expression of E-cadherin increased Bcl-2 promoter activity as did forced expression of HOXA1. The E-cadherin-stimulated increase in Bcl-2 promoter activity at full confluence was abrogated by siRNA-mediated depletion of HOXA1 (Fig. 11). The E-cadherin-stimulated increase in Bcl-2 gene transcription is therefore dependent on HOXA1 in accord with HOXA1-mediated cell survival stimulated by E-cadherin at confluence.

We also examined the effect of confluence on MCF-7 cell cycle progression by examination of the nuclear incorporation of 5'-bromo-2'-deoxyuridine (BrdUrd). MCF-7 cells at full confluence did not exhibit a significantly higher percentage of nuclear BrdUrd incorporation compared with MCF-7 cells at subconfluence (Fig. 12). Thus, E-cadherin

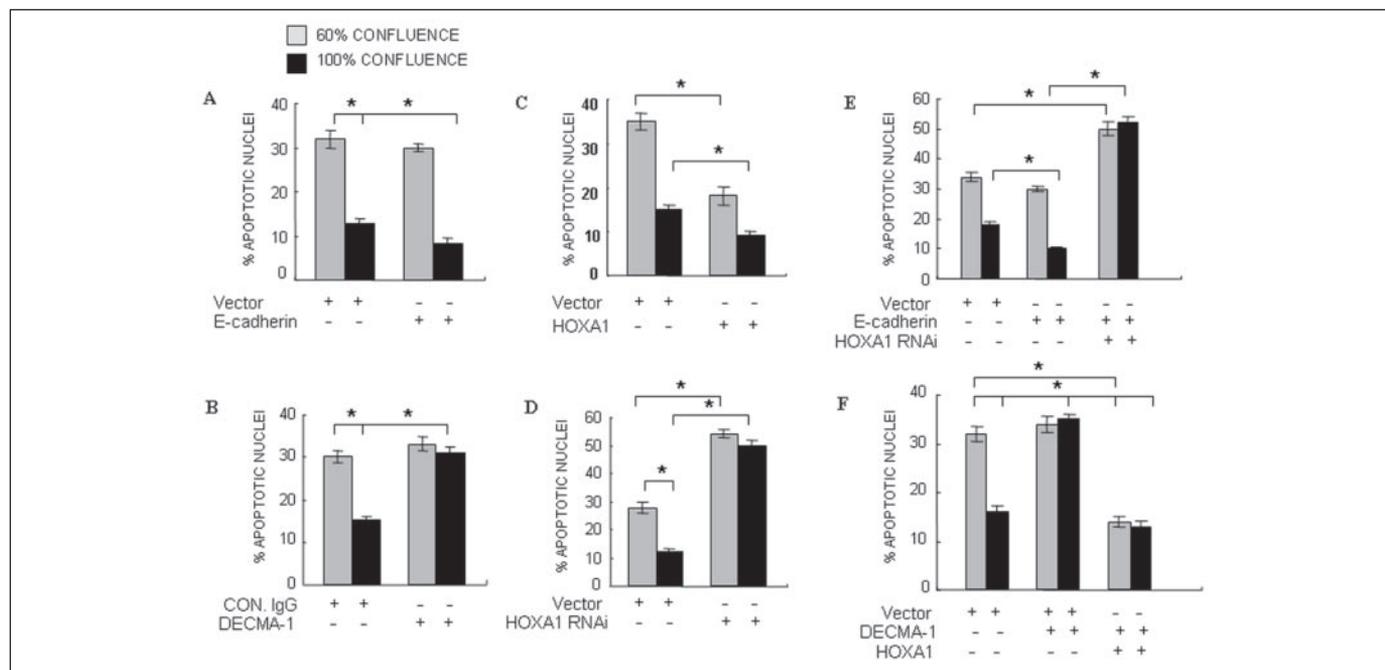
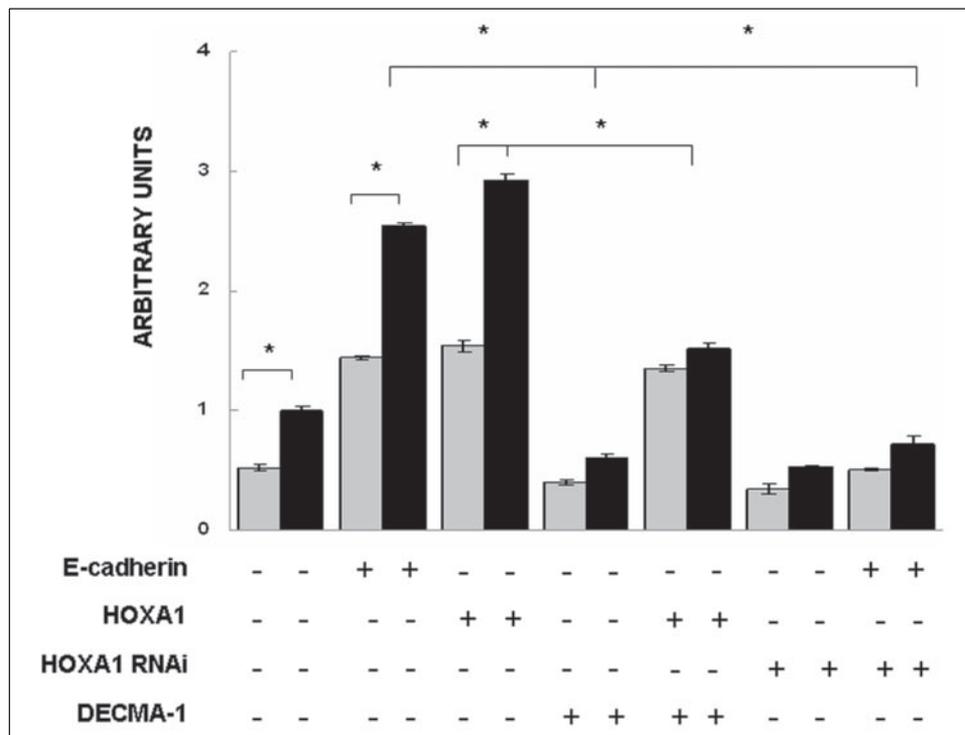


FIGURE 10. **Increased HOXA1 expression by E-cadherin-activated signaling decreases apoptotic cell death in human mammary carcinoma cells.** MCF-7 cells were cultured at subconfluence (60% confluence) and full confluence (100% confluence) and transiently transfected with an E-cadherin expression plasmid (A and E), HOXA1 expression plasmid (C and F), HOXA1 RNAi (E and F), or treated with DECMA-1 (E-cadherin function-blocking antibody) (B and F) and apoptotic cell death determined as detailed under "Experimental Procedures." Expression of E-cadherin at confluence increased cell survival (A and E); DECMA-1 prevented confluence-dependent cell survival (B and F); HOXA1 RNAi prevented the increased cell survival at full confluence (D), even with forced expression of E-cadherin (E). Blocking of E-cadherin function prevented confluence dependent cell survival (B and F); and expression of HOXA1 rescued loss of E-cadherin function (F). *, $p < 0.01$.

FIGURE 11. **E-cadherin dependent stimulation of Bcl-2 promoter activity is HOXA1-dependent.** MCF-7 cells were transiently transfected with the respective plasmids as indicated with or without DECMA-1. Luciferase assays for determination of Bcl-2 promoter activity were performed as described under "Experimental Procedures" and normalized to constitutive β -galactosidase activity. *, $p < 0.01$.



ligation does not result in a proliferative signal in MCF-7 cells in monolayer culture. Depletion of HOXA1 by use of siRNA to HOXA1 diminished BrdUrd incorporation in both subconfluent and confluent culture in accord with our previous demonstration that HOXA1 stimulates, albeit minimally, MCF-7 cell cycle progression (21).

Increased HOXA1 Expression by E-cadherin-activated Signaling Increases Anchorage-independent Proliferation in Human Mammary

Carcinoma Cells—E-cadherin has previously been demonstrated to mediate aggregation-dependent survival of epithelium-derived cells (27, 28, 31). To determine the effect of increased HOXA1 expression by E-cadherin-activated signaling on anchorage-independent proliferation in MCF-7 cells, we examined the growth of non-aggregated and aggregated MCF-7 cells in suspension culture. Aggregated MCF-7 cells increased in number significantly greater than non-aggregated MCF-7

cells in suspension culture and after 5 days had approximately doubled in number (Fig. 13A). In contrast, non-aggregated MCF-7 cells exhibited a slow decline in number and at 5 days were ~50% of the original plating number. Depletion of HOXA1 levels by HOXA1 siRNA inhibited anchorage-independent growth of aggregated MCF-7 cells (Fig. 13B). Furthermore, DECMA-1 prevented anchorage-independent growth of aggregated MCF-7 cells but not of aggregated MCF-7 cells with forced stable expression of HOXA1 (Fig. 13C). Thus, increased HOXA1 expression consequent to E-cadherin-activated signaling is required for anchorage-independent proliferation of human mammary carcinoma cells.

DISCUSSION

We have demonstrated herein that E-cadherin homophilic ligation stimulates the expression of HOXA1 in a Rac1-dependent manner. HOXA1 is subsequently utilized by E-cadherin to execute a program of cell survival and permits E-cadherin to stimulate anchorage-independent proliferation of human mammary carcinoma cells. We have therefore identified HOXA1, a powerful human mammary epithelial oncogene (21), as an effector molecule of E-cadherin.

In the normal mammary gland E-cadherin is expressed by the luminal epithelial cells and is localized to sites of intercellular contact (43). A large number of studies have demonstrated a loss of E-cadherin in the majority of lobular carcinomas of the mammary gland (44–46). However, this type of carcinoma represents a minority of carcinomas derived from the mammary epithelial cell. In contrast to lobular carcinomas, most ductal carcinomas retain E-cadherin expression, although the actual level of expression may vary (44–47). In this regard, E-cadherin-activated HOXA1 will be a mechanism to provide a contact-dependent

survival signal independent of integrins (25). In general, E-cadherin expression is higher in well differentiated and less invasive tumors as opposed to poorly differentiated or invasive tumors (48). Although E-cadherin expression does not correlate with lymph node status of mammary tumors (49), a number of experimental studies indicate a role of E-cadherin in metastatic progression. For example, forced expression of E-cadherin in E-cadherin negative mammary carcinoma cells suppresses metastasis in a xenograft model (50) and tamoxifen restores the function of E-cadherin in human mammary carcinoma cells and suppresses their invasive phenotype (51). Expression of HOXA1 has also been demonstrated to increase in neoplastic lesions of the mammary gland (17, 18), although comprehensive analysis of the tumor stages or subtypes was not performed. It is well documented that mammary carcinoma cell behavior may be altered by autonomous expression of molecules involved in cell survival and/or proliferation (52). E-cadherin autonomous expression of HOXA1 may offer a molecular strategy for the cell to dispense with E-cadherin, opening the possibility for cells to down-regulate E-cadherin to undergo epitheliomesenchymal transition (EMT), invasion and potential metastasis while remaining resistant to anoikis. Indeed, our unpublished observations³ have demonstrated that HOXA1 itself does not stimulate EMT in human mammary carcinoma cells nor any morphological or functional changes consequent to EMT. Furthermore, although we observed a positive correlation between the expression level of E-cadherin and HOXA1 in normal/benign mammary tissue in the Oncomine data base, no such correlation was observed in samples of invasive mammary carcinoma within the same data base. Thus, E-cadherin utilizes HOXA1 specifically for cell survival and not for maintenance of the epithelial phenotype and potential autonomous expression of HOXA1 will release the mammary carcinoma cell from the requirement of E-cadherin to prevent anoikis. Alternatively, dysregulation of Rac1 in neoplastic progression may contemporaneously down-regulate E-cadherin-mediated intercellular adhesion (53) and up-regulate HOXA1, providing carcinoma cells with the opportunity to escape anoikis while undergoing EMT.

We have previously demonstrated that forced expression of HOXA1 dramatically enhances anchorage-independent proliferation of mammary carcinoma cells and allows anchorage-independent growth of non-transformed human mammary epithelial cells (21). The effects of HOXA1 on increasing cell survival and preventing anoikis required the transcriptional up-regulation of Bcl-2 via the P1 promoter (21). Thus, Bcl-2 knockdown or inhibition of Bcl-2 function prevented the cell survival-promoting actions of HOXA1 in the mammary carcinoma cell. In

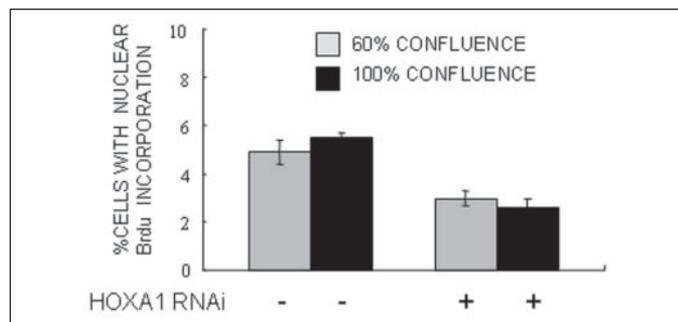


FIGURE 12. **Effect of cell confluence on mammary carcinoma cell cycle progression.** MCF-7 cells transiently transfected with vector or HOXA1 siRNA were cultured at subconfluence (60% confluence) and full confluence (100% confluence) and nuclear incorporation of BrdUrd determined as described under "Experimental Procedures."

³ X. Zhang and P. E. Lobie, unpublished observations.

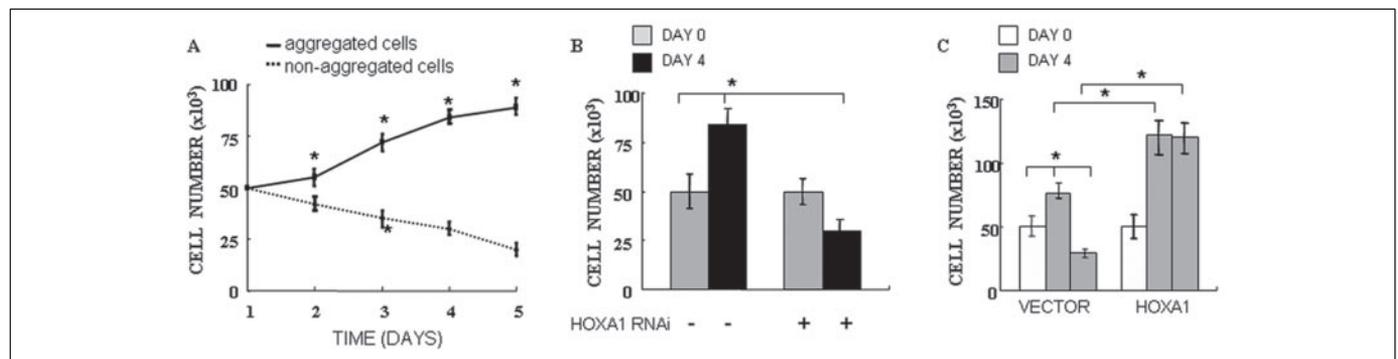


FIGURE 13. **Increased HOXA1 expression by E-cadherin-activated signaling increases anchorage-independent proliferation of human mammary carcinoma cells.** Anchorage-independent growth of aggregated and separate MCF-7, MCF7-VECTOR, and MCF7-HOXA1 cells in bacteriological dishes were performed as described under "Experimental Procedures." Aggregated cells increased in number in suspension culture (A). Use of HOXA1 RNAi inhibited anchorage-independent growth of aggregated MCF-7 cells (B), and DECMA-1 prevented anchorage-independent growth of MCF7-VECTOR but not MCF7-HOXA1 cells (C). *, $p < 0.01$.

E-cadherin Regulates HOXA1

addition, forced expression of HOXA1 produced resistance to apoptosis induced by doxorubicin, another effect mediated by the HOXA1-stimulated increase in Bcl-2 (21). In this regard it is interesting that MCF-7 cells exhibit relative chemoresistance when grown as three-dimensional spheroids as opposed to culture as two-dimensional monolayers (54). Functional disruption of E-cadherin was reported to increase the chemosensitivity of MCF-7 cells in three-dimensional culture indicative that E-cadherin-mediated intercellular contact produced chemoresistance (54). Decreased chemosensitivity of ovarian carcinoma cells grown in spheroids as opposed to monolayer culture has been demonstrated to be associated with enhanced expression of Bcl-XL (55). E-cadherin-mediated intercellular contact has recently been demonstrated to increase Bcl-2 in squamous cell carcinoma by EGF-R-dependent activation of p44/42 MAP kinase (28). In this regard it is interesting that we have demonstrated that HOXA1 itself regulates the expression of components of the pathway used for activation of p44/42 MAP kinase, including Grb2 and MEK1, and results in dramatically elevated p44/42 MAP kinase-dependent transcriptional activity.⁴ Thus, E-cadherin regulation of HOXA1 will potentially prime or sensitize the cell to activators of the p44/42 MAP kinase pathway. E-cadherin may potentially utilize HOXA1 to increase expression of Bcl-2 analogous to the use of HOXA1 by autocrine human growth hormone (19) to transcriptionally activate molecules, including Bcl-2, required for cell survival.

Classic cadherins can regulate cell signaling via a number of general mechanisms. These include serving as upstream receptors to activate signaling pathways and mediating juxtacrine signaling (29). Our data show that not only is E-cadherin necessary for cell-cell contact to stimulate HOXA1, but homophilic ligation itself, induced using recombinant E-cadherin adhesive ligands, is sufficient to induce the expression of this key transcription factor. Our current data therefore identifies HOXA1 as a critical transcriptional target for "direct" cadherin-activated cell signaling. A number of signaling pathways can be activated by cadherin homophilic ligation, including small GTPases of the Rho family, lipid kinases, and receptor tyrosine kinases. Notably, a key pathway activated by classic cadherins such as E-, C-, and N-cadherin (42, 56, 57) involves the small GTPase, Rac. This is best understood to regulate the cortical actin cytoskeleton at cadherin adhesive contacts. However, Rac is also known to regulate transcriptional events (29), consistent with our observation that HOXA1 expression depended on Rac, but not phosphatidylinositol 3-kinase, signaling. This further defines HOXA1 as a transcriptional target of the cadherin-activated Rac signaling pathway. Interestingly, inhibition of Rac1 by a newly developed small molecule inhibitor has been demonstrated to inhibit anchorage-independent proliferation of human prostatic carcinoma cells (58), an effect potentially mediated by HOXA1.

The mammary gland is one of very few organs that are subjected to substantial postnatal development; cycles of growth, differentiation, apoptosis, regression, and remodeling are maintained almost during the lifetime of the organism (59). In this regard, the association between E-cadherin and HOXA1 is interesting given the pivotal developmental role of E-cadherin (60, 61) and members of the homeobox family of genes in general (10, 11). E-cadherin is expressed at all stages of mammary development (43). It is predominantly localized to ductal luminal cells and end bud body cells. Exposure of end buds to a function blocking E-cadherin antibody disrupts the body epithelium (62). In addition, mammary-specific inactivation of E-cadherin resulted in massive cell death at parturition to produce a gland that resembled the involuted

gland normally observed after weaning (63). Similarly, deletion of three paralogous homeobox genes, Hoxa9, Hoxb9, and Hoxd9, produce hypoplasia of the mammary gland during pregnancy and post parturition (64). Although E-cadherin has been only demonstrated to date to regulate HOXA1 (this study), it is likely that E-cadherin will regulate other members of the HOX family to direct specific developmental programs in the mammary gland. One potential physiological function of E-cadherin-regulated HOXA1 may be to provide the capacity for luminal filling during virgin ductal development where resistance to anoikis would be required. Another may be to provide cell-survival functions important for initiation and maintenance of lactation. In any case, the ability of E-cadherin to regulate HOXA1 expression for anchorage-independent growth of mammary carcinoma cells will be a pathological recapitulation of a normal developmental function.

In conclusion, we have demonstrated that increased HOXA1 expression and transcriptional activity upon formation of intercellular contacts in mammary carcinoma cells was mediated by E-cadherin activation of Rac1. E-cadherin utilized HOXA1 to increase cell survival and facilitate anchorage-independent proliferation. HOXA1 is therefore a downstream effector molecule of E-cadherin-directed signaling.

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