Oncogenic Transformation of Human Mammary Epithelial Cells by Autocrine Human Growth Hormone

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Abstract

The human growth hormone (*hGH*) gene is expressed in the normal human mammary epithelial cell and its expression increases concomitant with the acquisition of proliferative lesions. Herein we demonstrate that autocrine production of hGH in human mammary carcinoma cells dramatically enhances anchorage-independent growth in a Janus kinase 2–dependent manner. Forced expression of the *hGH* gene in immortalized human mammary epithelial cells increased proliferation, decreased apoptosis, altered the cellular morphology and resulted in oncogenic transformation. Autocrine hGH was therefore sufficient to support anchorage-independent growth of immortalized human mammary epithelial cells and tumor formation *in vivo*. Moreover, autocrine hGH disrupted normal mammary acinar architecture with luminal filling and deregulated proliferation in three-dimensional epithelial cell culture. Autocrine hGH utilized homeobox A1 to govern the transcriptional program required for autocrine hGH–stimulated oncogenic transformation of human mammary epithelial cells, including transcriptional up-regulation of *c-Myc*, *cyclin D1*, and *Bcl-2*. Forced expression of a single orthotopically expressed wild-type gene is therefore sufficient for oncogenic transformation of the immortalized human mammary epithelial cell. (Cancer Res 2005; 65(1): 317-24)

Introduction

It has been hypothesized that cancer arises from a stepwise accumulation of genetic changes that liberates neoplastic cells from the homeostatic mechanisms that govern normal cell proliferation (1). A contrasting but complementary hypothesis has recently been proposed that deregulation of proliferation, together with a reduction in apoptosis, creates a platform that is both necessary and sufficient for development of cancer (2). Such a hypothesis is reinforced by the fact that many mechanisms that drive cell proliferation also possess the potential to trigger or sensitize a cell to apoptosis (3). Presumably the two genetic elements (the SV40 large-T oncoprotein and an oncogenic allele of H-*ras*), identified by Hanahan and Weinberg to be sufficient for oncogenic transformation of human mammary epithelial cells (1), in addition to *hTERT* required for cellular immortalization, perform a functionally equivalent task to the required platform of deregulated cell proliferation and survival.

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 (4). Growth hormone is obligatory for normal pubertal mammary gland development by acting on both the mammary stromal and epithelial components to result in ductal elongation and the differentiation of ductal epithelia into terminal end buds (5, 6). Expression of the human growth hormone (*hGH*) transgene in mice results in precocious development of the mammary gland (7, 8) and the development of neoplasia (8). Conversely, spontaneous or experimentally engineered functional deficiency of growth hormone (9–12) results in severely impaired mammary gland development and virtual resistance to the spontaneous development of hyperplastic alveolar nodules (9) and chemically induced mammary carcinogenesis (10, 12). Similarly, in a primate model, hGH administration results in marked hyperplasia of the mammary gland with an increased epithelial proliferation index (13). We have demonstated the expression of the *hGH* gene in the normal human mammary gland epithelium (14). Increased epithelial expression of the *hGH* gene is associated with the acquisition of pathologic proliferation and the highest level of *hGH* gene expression is observed in metastatic mammary carcinoma cells (14). The autocrine production of hGH in human mammary carcinoma cells has been shown to result in a hyperproliferative state (15) with an aggressive cellular morphology (16).

In the present study, we demonstate that autocrine production of *hGH* in immortalized human mammary epithelial cells concomitantly enhances proliferation and offers protection from apoptosis; forming the basis for abnormal mammary acinar morphogenesis, oncogenic transformation, and tumor formation *in vivo*. Thus, simple forced expression of a single orthotopically expressed wild-type gene is sufficient for oncogenic transformation of the immortalized human mammary epithelial cell.

Materials and Methods

Cell Lines, Cell Culture, and Transfections. The MCF-7, MCF-10A, and NIH-3T3 cell lines were obtained from the American Type Culture Collection. MCF-7-hGH expressing wild-type *hGH* gene and its cognate control MCF-7-MUT were established as described (15). A detailed description of the characterization of these cell lines has been published previously (15, 16). pcDNA3 vector alone and pcDNA3-hGH vector were stably transfected into NIH-3T3 cells or MCF-10A cells by use of Effectene (Qiagen, Inc., Valencia, CA). Expression of *hGH* mRNA in the stable cell lines were confirmed by both reverse transcription–PCR (RT-PCR) and ELISA as described previously (15, 16).

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5′-Bromo-2′-Deoxyuridine Incorporation Assay and Measurement of Apoptosis. Mitogenesis was directly assayed by measuring the incorporation of bromodeoxyuridine (16). Apoptotic cell death was measured by fluorescent microscopic analysis of cell DNA staining patterns with Hoechst 33258 as previously described (16).

Cell Behavior and Morphogenesis Assays in Matrigel. Tissue culture dishes were coated with Matrigel (BD Bioscience, Franklin Lakes, NJ) at 37°C for 30 minutes before adding 1×10^5 vector- or *hGH*-transfected MCF-10A cells. The behavior of both cell lines was assessed and digitally recorded at 6-hour intervals using an inverted light microscope (Nikon TE200, Nikon, Tokyo, Japan). The three-dimensional culture of MCF-10A cells on basement membrane was done as described (17). Assay medium with 2% Matrigel was replaced every 4 days.

Confocal Laser Scanning Microscopic Analysis and Image Acquisition. Acinar structures were fixed in 2% formalin at room temperature for 25 minutes and permeabilized in 0.5% Triton X-100 in PBS for 10 minutes at 4°C. Except for this minor modification in fixation and permeabilization, the immunostaining of acinar structures was carried out as described (17). Anti-GM130 was purchased from BD Transduction (San Diego, CA), anti–Ki-67 from Zymed (San Francisco, CA), anti–α_6 integrin from Chemicon (Temecula, CA), phospho-ERM from Cell Signaling (Beverley, MA), and TO-PRO3 from Molecular Probes (Eugene, OR). Confocal analyses were done using the Bio-Rad MRC1024 confocal microscopy system (Bio-Rad, Hercules, CA).

Reverse Transcription-PCR. Extraction of total RNA and RT-PCR assay were done as described (18). Sequences of the oligonucleotide primers used for RT-PCR are as follows: 5′-AAA GGC CCC CAA GGT AGT TA-3′ and 5′-TTC TTT TAT GCC CAA AGT CCA-3 for *c-Myc*; 5′-CCC TCG GTG TCC TAC TTC AAA-3′ and 5′-CAC CTC CTC CTC CTC CTC TTC-3′ for *Cyclin D1*; 5′-ATG GCG CAC GCT GGG AGA AC-3′ and 5′-GCG GTAG CGG CGG GAG AAG T-3′ for *Bcl-2*; and 5′-ATG ATA TCG CCG CGC TCG-3′ and 5′-CGC TCG GTG AGG ATC TTC A-3′ for β-*Actin*.

Soft Agar Colony Formation and Suspension Culture Assays. Anchorage-independent growth assays, including suspension culture, were performed as previously described (19). For soft agar colony formation assay, MCF-7 cell and its derived cell lines were cultured in six-well plates with a 0.35% agar layer with or without 50 nmol/L *hGH* ± 50 μmol/L Janus-activated kinase (JAK) 2–specific inhibitor AG490 or Src kinase inhibitor PP1 and PP2 (20, 21). The MCF10A-vector and MCF10A-hGH cell soft agar colony formation assays were processed the same as above, except that the medium was changed to DMEM/Ham's F-12 medium containing appropriate components as described (19), and 10^4 cells were seeded to the middle layer of the soft agar. The plates were incubated for 10 days (for MCF-7 cells) or 14 days (for MCF-10A cells), after which the cultures were inspected and photographed.

Luciferase Reporter Assay. Transient transfection was performed with the respective luciferase constructs (18) and other expression constructs as appropriate were transfected in serum-free medium for 48 hours. Results were normalized to the level of β-galactosidase activity and protein concentration in the samples. The *c-Myc* promoter reporter plasmid was a kind gift of Dr. Hiroyoshi Ariga (Sapporo, Japan). The *Cyclin D1* promoter-luciferase construct (-1745CD1LUC) was a kind gift of Dr. Richard G. Pestell (Albert Einstein College of Medicine, New York, NY). *Bcl-2* P1 promoter reporter plasmid, *EphA2-r42B-LUC* reporter plasmid, and the PBX1 expression vector have been described previously (19).

Small Interfering RNA Preparation and Transfection. The small interfering RNA (siRNA)-encoding complementary single-stranded oligonucleotides were designed using the computer program available on the Web site of Ambion (www.ambion.com). Four different target sequences were initially chosen for each siRNA design. Both RT-PCR and luciferase reporter assay were performed to assess gene silencing efficiencies of different siRNA constructs (data not shown). Only siRNAs with >70% efficiency in reduction of the selected genes were chosen for subsequent functional studies. The siRNA sequence used targeting human *HOXA1* is 5′-GTT GTG GTC CAA GCT ATG G-3′; human *c-Myc*, 5′-TCG ATG TTG TTT CTG TGG A-3′; and human *Bcl-2*, 5′-TGA GTG ACG AGT TTG TGG A-3′. Human *Cyclin D1* siRNA was purchased from Ambion (Austin, TX). Control oligonucleotides corresponded to the inverse sequences. Oligonucleotides encoding siRNAs (Invitrogen, San Diego, CA) were annealed and ligated into *pSilencer*-U6 vector (Genscript, Piscataway, NJ). 0.2 μg of siRNA/vector control per well was transfected into six-well plates by use of Effectene (Qiagen) for 24 to 48 hours. To exclude potential off target effects of the individual siRNA molecules used herein we carried out rescue experiments by transient forced expression of the corresponding targeted molecule (HOXA1, Bcl-2 and c-Myc). Bcl-2 and c-Myc expression vectors were a kind gift of Dr. Bert Vogelstein (Johns Hopkins University, Baltimore. MD). MCF-7 soft agar colony formation was used to indicate rescue and colony formation was performed essentially as described above for MCF-10A cells. Vector control (0.5 μg) or siRNA ± 5 μg expression vector (HOXA1, Bcl-2, or c-Myc) was transfected into MCF-7 cells in six-well plates by use of Effectene. Cells were subsequently plated in soft agar as described above in triplicate and colony formation determined. Colony number by vector, siRNA, and siRNA plus expression vector is (mean ± SD) 571 ± 22, 201 ± 14, and 770 ± 43 for HOXA1; 476 ± 45, 234 ± 30, and 439 ± 27 for Bcl-2; and 453 ± 50, 230 ± 13, and 451 ± 37 for c-Myc.

Xenograft Analyses. MCF-10A-vector (5×10^6) or MCF-10A-hGH cells were suspended in 100 μl PBS or Matrigel and injected into the first mammary (axillary) fat pad of 3- to 4-week-old athymic (*nu/nu*) mice (The Jackson Laboratory, West Grove, PA), which simultaneously received a 60-day release pellet containing 0.72 mg of 17β-estradiol (Innovative Research of America, Southfield, MI) (19). The latency of the tumor from the nude mice in this study is around 3 weeks and tumors were harvested 6 weeks after inoculation. 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 and stained with H&E to assess morphology. Total RNA extraction and RT-PCR for *hGH* were done by use of fresh lung and liver tissue to detect possible metastasis of the tumors (19).

Results

Autocrine hGH Production by Mammary Carcinoma Cells Enhances Anchorage-Independent Growth. One characteristic of oncogenically transformed cells is the capacity for anchorage-independent growth (1–3). Autocrine production of hGH in MCF-7 cells dramatically enhanced anchorage-independent growth as indicated by colony formation in soft agar (Fig. 1*A*) and growth in suspension culture (Fig. 1*C*). Furthermore, the individual colony size was markedly increased by autocrine hGH (Fig. 1*B*). Exogenously applied recombinant hGH (50 nmol/L applied daily) did not enhance colony formation in soft agar by MCF-7 cells (Fig. 1*A*) and, surprisingly, slightly abrogated the effect of autocrine hGH on colony formation. Cellular effects of hGH are primarily mediated by stimulation of JAK2 activity (22). The enhanced colony formation by MCF-7 cells due to autocrine hGH production was completely inhibited by a specific inhibitor of JAK2 (Fig. 1*D*), whereas the basal level of MCF-7 cell colony formation was not significantly affected by inhibition of JAK2. Src kinase–specific inhibitors PP1 and PP2 (or the inactive structural analogue PP3) did not inhibit autocrine hGH–stimulated colony formation (data not shown). Thus, autocrine hGH enhancement of mammary carcinoma cell anchorage-independent growth is JAK2 dependent.

Autocrine hGH Causes Oncogenic Transformation of Immortalized Human Mammary Epithelial Cells. To determine if forced expression of hGH would result in oncogenic transformation of human mammary epithelium we used the immortalized human mammary epithelial cell line MCF-10A.

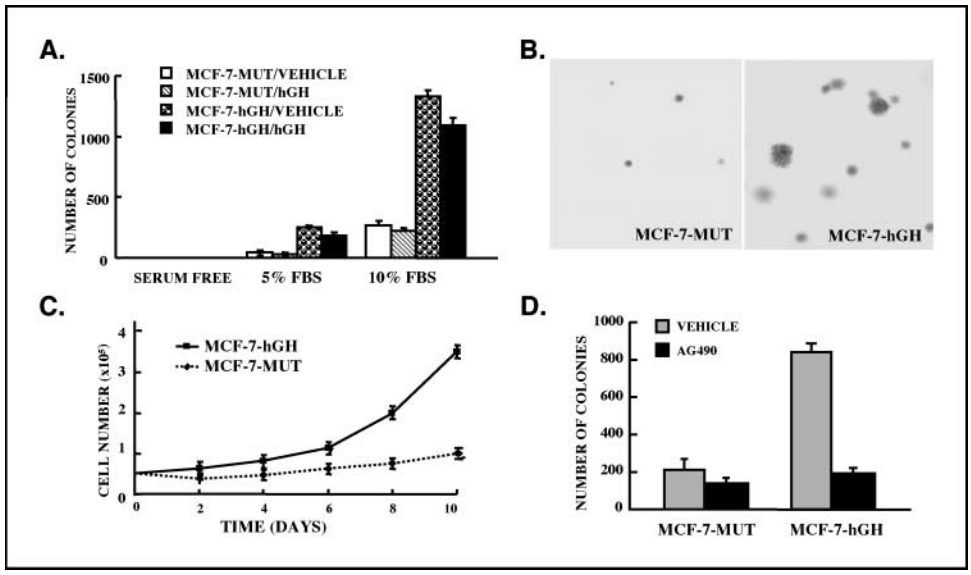

Figure 1. Autocrine hGH production by human mammary carcinoma cells results in enhanced anchorage-independent growth. *A,* soft agar colony formation in MCF-7-MUT or MCF-7-hGH (15) ± 50 nmol/L exogenous or hGH added daily. *B,* visualization of soft agar colony formation 10% fetal bovine serum by MCF-7-MUT and MCF-7-hGH cells as indicated. *C,* growth curve of MCF-7-MUT and MCF-7-hGH cells in suspension culture. Media contained 10% fetal bovine serum. *D,* effect of inhibition of JAK2 activity with 50 μmol/L AG490 on soft agar colony formation by MCF-MUT and MCF-hGH cells.

MCF-10A cells do not express the *hGH* gene nor secrete any significant amount of hGH (Fig. 2*A*). Stable transfection of MCF-10A cells with the complete *hGH* gene resulted in cellular hGH production and secretion of hGH from the cell (Fig. 2*B*). The concentration of hGH produced in the cell is similar to that reported for hGH content of human intraductal carcinoma[5] (14, 15). Autocrine production of hGH in this cell line both significantly increased mitogenesis (Fig. 3*A*) and abrogated apoptotic cell death as a consequence of serum deprivation (Fig. 3*B*) when compared to vector-transfected control cells. Consequently, autocrine production of hGH by MCF-10A cells resulted in an approximate 4-fold increase in cell number (data not shown). Microscopic evaluation revealed differences in morphology between MCF-10A cells with autocrine production of hGH compared to vector-transfected control cells. Vector-transfected MCF-10A cells displayed an epithelial morphology, with cell to cell interaction, whereas MCF-10A-hGH exhibited a mesenchymal spindle-shaped phenotype with limited intercellular contact (data not shown). MCF-10A-vector cells rapidly organized into spherical colonies when cultured on Matrigel (Fig. 3*C*). In contrast, MCF-10A-hGH cells cultured on Matrigel adopted a stellate organization (Fig. 3*C*) that was indistinguishable from the behavior of aggressive mammary carcinoma cell lines such as MDA-MB-231 and MDA-MB-435 (data not shown).

The morphologic changes observed in immortalized mammary epithelial cells due to autocrine production of hGH are reminiscent of oncogenic transformation. Another characteristic of the transformed phenotype is the capacity for anchorage-independent proliferation (3, 9). MCF-10A-hGH cells formed numerous colonies in soft agar, whereas MCF-10A-vector cells were largely ineffective in colonization of soft agar (Fig. 4*A* and *B*). Generation of two further stable clone sets of MCF-10A-vector and MCF-10A-hGH confirmed this observation. Application of exogenous hGH did not support soft agar colony formation by MCF-10A-vector cells and paradoxically significantly inhibited the ability of MCF-10A-hGH cells to colonize soft agar (Fig 4*B*). Use of the JAK2-specific inhibitor AG490 prevented soft agar colony formation by MCF-10A-hGH cells, indicative that oncogenic transformation stimulated by autocrine production of hGH required the activity of JAK2 (Fig. 4*C*).

Autocrine Production of *hGH* Results in the Filling of the Luminal Space in the Mammary Acinus. When immortalized human mammary epithelial cells (MCF-10A) are cultured *ex vivo* in Matrigel, a source of extracellular matrix akin to basement membrane, acinar structures resembling the *in vivo* morphology of the mammary gland are formed (17, 23, 24). The construction of these acini requires coordinated apoptosis to enable lumen formation and proliferative arrest of the remaining outer layer of mammary epithelial cells (17). In contrast, oncogenically transformed mammary epithelial cells form large, nonpolarized, undifferentiated colonies without lumina when grown in Matrigel (23). We used this *ex vivo* model to examine the effects of autocrine hGH on the architecture of structures formed by immortalized human mammary epithelial cells (24). Three-dimensional acinar structures were generated by plating MCF-10A cells as single cells in Matrigel. Phase-contrast (Fig. 5*A*) and confocal laser scanning microscopic analyses of acini labeled with TO-PRO3 revealed that

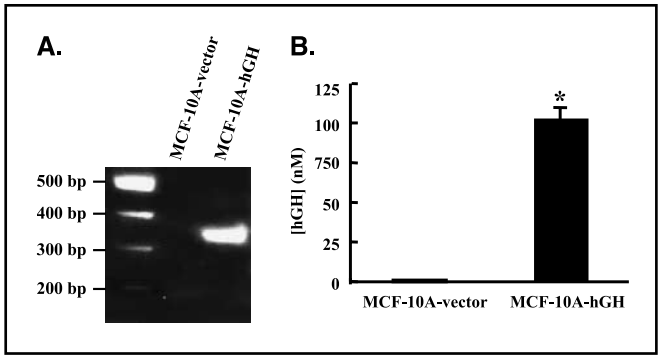

Figure 2. Generation of MCF-10A cells with autocrine expression of hGH (MCF-10A-hGH). MCF-10A-vector indicates vector-transfected control cells for comparison. Human growth hormone mRNA was detected by RT-PCR (*A*) and hGH secretion to the media was detected by ELISA (*B*).

[5] H.C. Mertani and P.E. Lobie, unpublished observations.

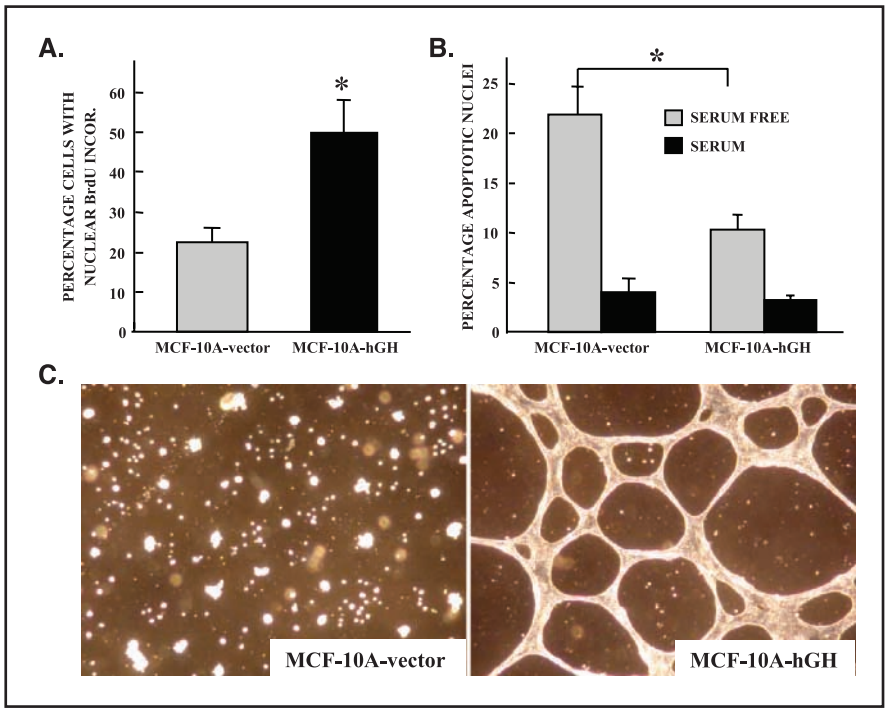

Figure 3. Autocrine production of hGH by immortalized human mammary epithelial cells regulates cell survival, proliferation, and morphology. *A,* effect of autocrine hGH production by MCF-10A cells on cell cycle progression indicated by nuclear incorporation of bromodeoxyuridine. *B,* effect of autocrine hGH production by MCF-10A cells on apoptosis induced by serum withdrawal. *C,* phase-contrast morphology of MCF-10A-vector and MCF-10A-hGH cells cultured on Matrigel.

the acinar units had basally localized nuclei and a hollow lumen (Fig. 5*C* and *E*). Localization of a basal surface marker (α_6 integrin; Fig. 5*C*), plasma membrane marker (phospho-ERM; Fig. 5*E*), and apical polarity marker (GM130; Fig. 5*C*) indicated that the acini formed consisted of polarized mammary epithelial cells. The small number of cells labeled with a proliferation marker (Ki-67; Fig. 5*C*) was consistent with proliferative arrest observed previously (17, 24). Autocrine production of hGH in immortalized human mammary epithelial cells resulted in the generation of large disorganized multiacinar structures with filled lumina (Fig. 5*B-F*), indicative of failure of luminal apoptosis. Autocrine production of hGH in immortalized human mammary epithelial cells also disrupted cellular polarization (Fig. 5*C* versus *D* and *E* versus *F*) and bypassed the proliferative arrest observed on normal acinar formation (Fig. 5*C* versus *D*). Labeling of cells with a lipophilic dye (DiI) and combination with unlabeled cells during the

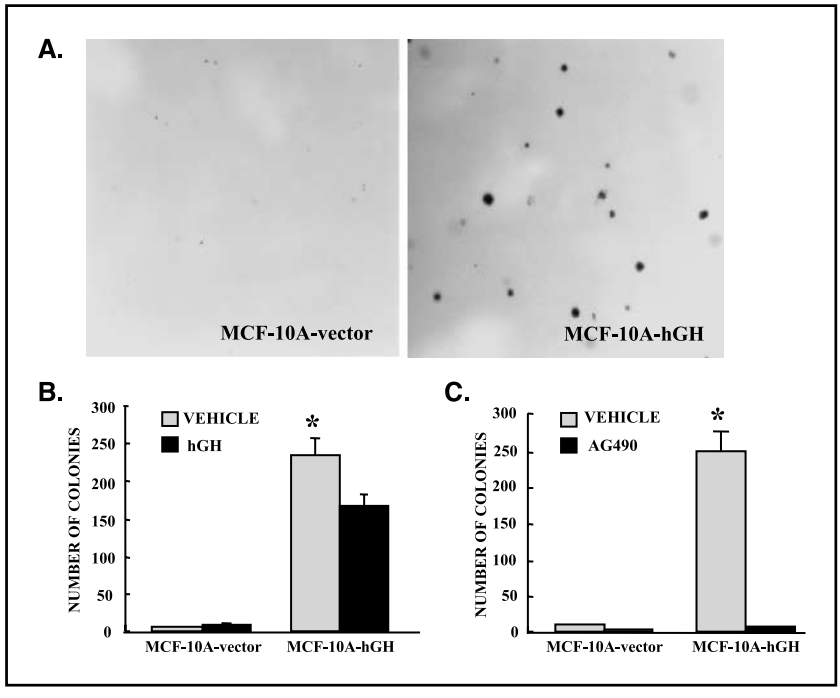

Figure 4. Autocrine hGH stimulation of anchorage-independent growth in immortalized human mammary epithelial cells is JAK2 dependent. *A,* visualization of soft agar colony formation by MCF-10A-vector and MCF-10A-hGH cells as indicated. *B,* soft agar colony formation in MCF-10A-hGH or MCF-10A-vector $\pm$ 50 nmol/L exogenous *hGH* added daily. *C,* effect of inhibition of JAK2 activity with 50 μmol/L AG490 on soft agar colony formation by MCF-10A-vector and MCF-10A-hGH cells. *Columns,* mean of triplicate determinations; *bars,* SD. *, $P < 0.01$.

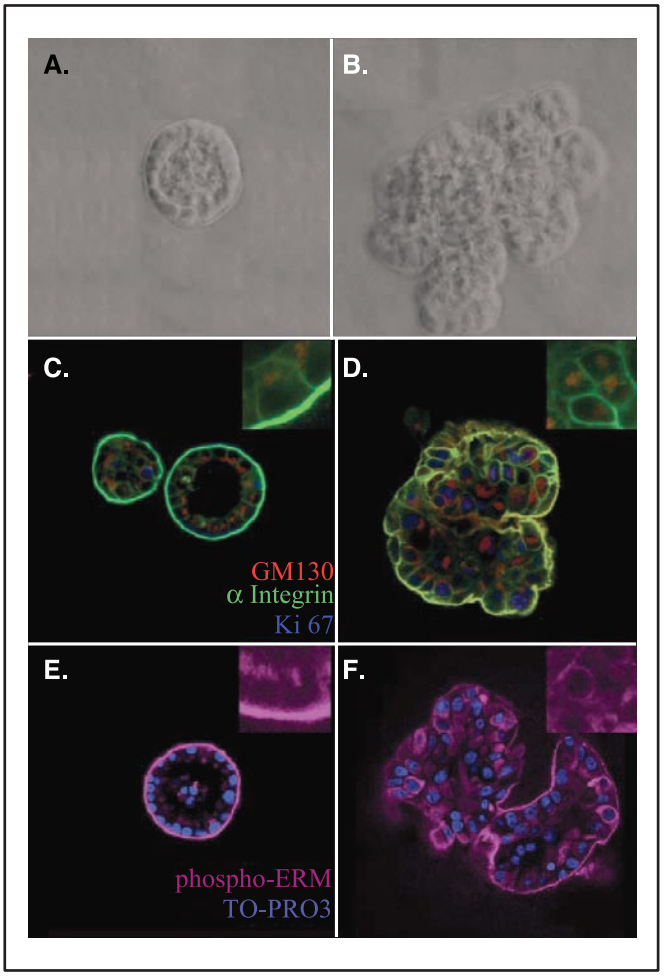

Figure 5. Autocrine hGH results in filling of the luminal space and loss of proliferative arrest in the human mammary acinus. Phase-contrast micrographs of vector (MCF-10A-vector; *A*) and hGH-expressing MCF-10A (MCF-10A-hGH; *B*) acini after 18 days in three-dimensional Matrigel culture. *C* to *F,* confocal laser scanning microscopic cross sections of the mammary acinar structures after 18 days in three-dimensional Matrigel culture. Confocal laser scanning microscopy was done with antibodies to α_6 integrin (*green*), GM130 (*red*), phospho-ERM (*purple*), and Ki-67 (*blue*). Nuclei were counterstained with TO-PRO3 (*blue*). *C* and *E,* MCF-10A-vector. *D* and *F,* MCF-10A-hGH cells.

morphogenesis assay showed that each acinus was derived from a single cell (data not shown).

Autocrine hGH Production in Immortalized Human Mammary Epithelial Cells Confers Tumorigenic Capacity. *In vitro* analyses of oncogenic transformation are not always concordant with tumorigenic potential *in vivo*. We therefore implanted both MCF10A-vector and MCF10A-hGH cells into the first mammary (axillary) fat pad of intact athymic female mice with use of either PBS or Matrigel as vehicle. Neither cell line produced palpable tumors when injected with PBS. However, MCF-10A-hGH cells with Matrigel injected as vehicle formed large palpable tumors (average size, 306 $\pm$ 43 mm^3) in the majority of injected animals (8/10), whereas MCF-10A-vector cells did not (0/10; Fig. 6*A*). The latency of macroscopic tumor appearance was approximately 3 weeks and tumors were harvested 6 weeks after injection. Necropsy revealed that the tumors were attached to the underlying axillary muscle and surrounded by a vascular fibrous capsule (Fig. 6*B*). Histologically, the neoplastic cells were locally invasive and associated with fibrous connective tissue (Fig. 6*C*). The cells exhibited moderate cytoplasmic and nuclear pleomorphism and formed a solid mass. Necropsy of female athymic mice given injections of MCF-10A-vector cells failed to identify any growth. Macroscopic and histologic examination of lung and liver failed to identify metastatic extension of the implanted MCF-10A-hGH cells (data not shown). Mammary carcinoma cells derived from the *in vivo* growth of MCF-10A-hGH cells expressed *hGH* at the same level as the injected cell, indicative of phenotypic retention (data not shown).

Autocrine hGH Oncogenically Transforms NIH-3T3 Cells. We next wished to determine if oncogenic transformation by autocrine production of hGH was a generalized phenomenon and not particular to the human mammary epithelial cell. NIH-3T3 cells are widely used in studies of oncogenic transformation (1, 25) and are responsive to hGH stimulation (20, 21, 26). We therefore generated stable NIH-3T3 transfectants with autocrine expression of hGH (data not shown). Autocrine production of hGH by NIH-3T3 cells resulted in dramatic foci formation and robust soft agar colony formation (Fig. 7*A* and *B*); two indicators of oncogenic transformation of NIH-3T3 cells not observed in vector-transfected control cells. Concordant with this observation, autocrine production of hGH stimulated a dramatic increase in NIH-3T3 cell number compared to the vector-transfected control (Fig. 7*C*).

Both Proliferative and Cell Survival Gene Transcription Is Required for Oncogenic Transformation by Autocrine *hGH*. Activation of signaling pathways resulting in oncogenic transformation is associated with transcriptional regulation of genes required for cell cycle progression and cell survival (3, 9). *c-Myc* and *Cyclin D1* are required for mammary epithelial cell cycle progression, whereas *Bcl-2* is required for mammary epithelial cell survival (1, 2, 27). c-Myc, cyclin D1, and Bcl-2 have all been reported to be overexpressed in breast cancer biopsies (28). Autocrine hGH increased the transcription and subsequent mRNA level and protein expression of *c-Myc*, *Cyclin D1*, and *Bcl-2* in human mammary epithelial cells (Fig. 8*A-C*). Transient transfection of siRNA to *c-Myc*, *Cyclin D1*, or *Bcl-2* significantly abrogated the

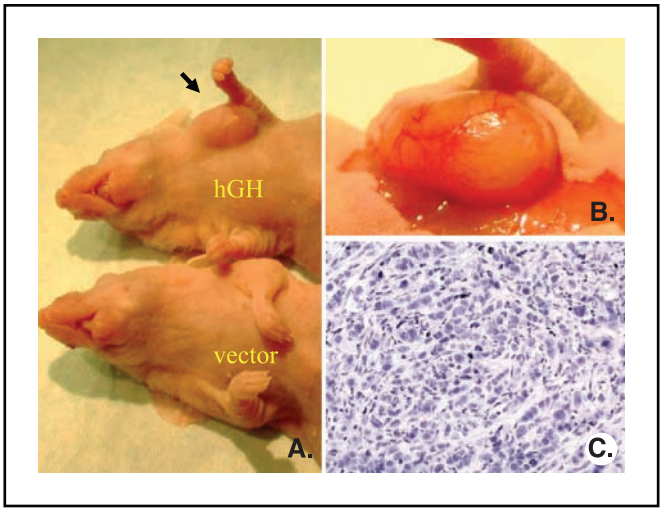

Figure 6. Autocrine hGH production in immortalized human mammary epithelial cells results in tumor formation *in vivo*. *A,* MCF-10A-hGH cells implanted into the first mammary fat pad of athymic female mice form a large visible tumor mass. *B,* tumor mass visualized on necroscopy. *C,* histologic appearance of the tumor visualized with H&E.

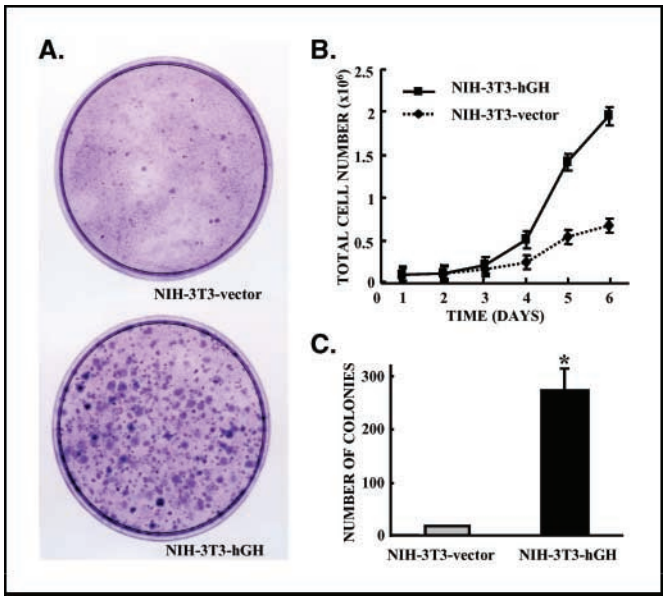

Figure 7. Transfection of the *hGH* gene into NIH-3T3 cells stimulates proliferation and oncogenic transformation. *A,* foci formation in NIH-3T3 cells stably transfected with the *hGH* gene or a control vector as described. *B,* monolayer growth curve of NIH-3T3-vector and NIH-3T3-hGH cells. *C,* soft agar colony formation by NIH-3T3-vector and NIH-3T3-hGH cells. *Columns,* mean of triplicate determinations; *bars,* SD. *, $P < 0.01$.

ability of autocrine hGH to stimulate colony formation in soft agar (Fig. 8*E*); the extent consistent with the efficiency of transfection (data not shown). Thus, activation of genes required for both proliferation and cell survival is necessary for oncogenic transformation of immortalized human mammary epithelial cells by autocrine hGH.

***HOXA1* Governs the Transcriptional Program of Autocrine *hGH* Required for Oncogenic Transformation.** We have recently reported that an autocrine hGH–regulated gene, *HOXA1*, is itself a powerful human mammary epithelial oncogene (19). Homeobox genes often govern higher order genetic switches that determine cell fate by coordination of regulatory pathways (29). Construction and transfection of an siRNA to *HOXA1* significantly impaired the ability of autocrine hGH to increase HOXA1-mediated transcription (Fig. 8*D*). Knockdown of *HOXA1* also significantly inhibited the ability of autocrine hGH to stimulate transcription of the *c-Myc*, *Cyclin D1*, and *Bcl-2* genes (Fig. 8*D*), indicative that autocrine hGH regulation of these genes is predominantly governed by *HOXA1*. Basal transcription of *c-Myc, CyclinD1*, and *Bcl-2* was not affected by the diminished expression of HOXA1 (data not shown). Transient transfection of the same *HOXA1* siRNA construct also dramatically abrogated the ability of autocrine *hGH* to stimulate colony formation by human mammary epithelial cells in soft agar (Fig. 8*E*). Thus, autocrine hGH is using *HOXA1* to govern the expression of a transcriptional program, resulting in proliferation, cell survival, and ultimately oncogenic transformation of immortalized human mammary epithelial cells.

Discussion

We show here that autocrine production of hGH, by itself, is sufficient to oncogenically transform the immortalized human mammary epithelial cell with consequent tumor formation *in vivo*. This is remarkable given that other oncogenes, such as *ras*, *HER2*, *TC21*, and *Cyclin D1* are insufficient to convey tumorigenic potential on MCF-10A cells (19, 30). This is presumably because many mechanisms that drive cell proliferation also possess the potential to trigger or sensitize a cell to apoptosis (2, 3). Established examples include activation of the Ras/Raf pathway and deregulated expression of c-Jun, c-Myc, and E2F transcription factors (2). Moreover, other versions of this so-called antagonistic pleiotropy (2) exist, such as the innate capacities of activated Raf and Ras

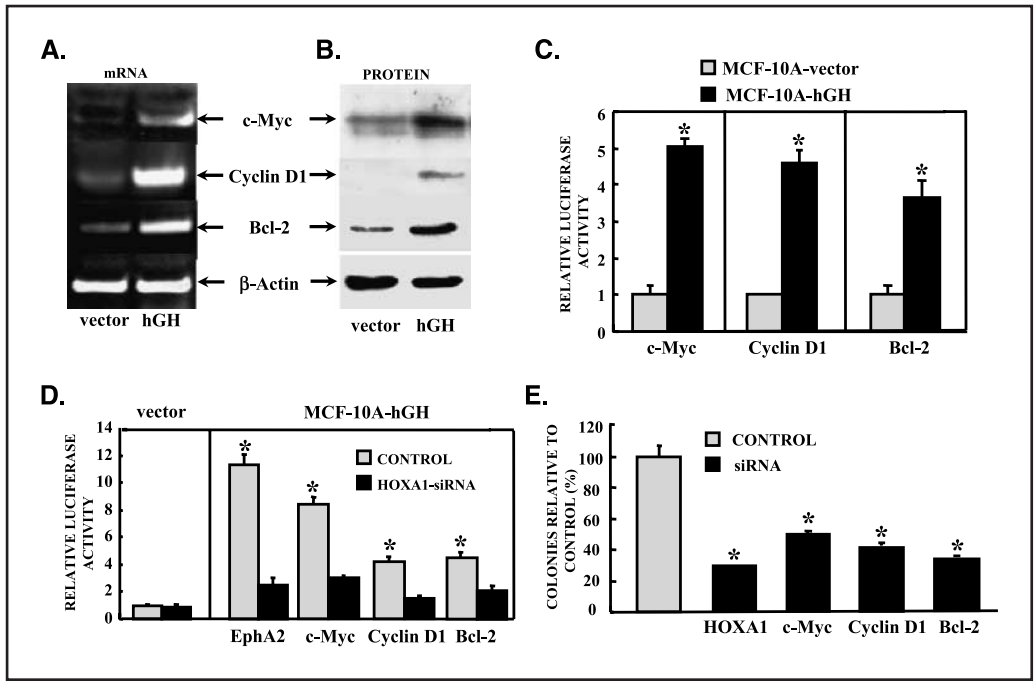

Figure 8. Autocrine hGH concomitantly activates a proliferative and antiapoptotic transcriptional program for anchorage-independent growth. RT-PCR (*A*) and Western blot analysis (*B*) of c-Myc, Cyclin D1, and Bcl-2 expression in MCF-10A-vector and MCF-10A-hGH cells. *C,* MCF-10A-vector and MCF-10A-hGH cells were transiently transfected with *c-Myc*, *Cyclin D1*, and *Bcl-2* promoter reporter plasmids for determination of autocrine hGH–stimulated transcriptional activity. *D,* MCF-10A-vector and MCF-10A-hGH cells were transiently transfected with *EphA2*, *c-Myc*, *Cyclin D1*, and *Bcl-2* promoter reporter plasmids in the presence or absence of *HOXA1*-siRNA plasmid. *E,* quantification of soft agar colony formation by MCF-10A-hGH in the presence of control siRNA construct or siRNA construct for *HOXA1*, *c-Myc*, *Cyclin D1*, or *Bcl-2*. *Columns,* mean of triplicate determinations; *bars,* SD. *, $P < 0.01$.

oncoproteins to trigger permanent growth arrest (2, 3) or the inhibitory action of the apoptosis suppressor Bcl-2 on cell proliferation (2). Therefore, uncontrolled cell proliferation, with obligate suppression of compensatory apoptosis, is required to create a platform that will be both necessary and sufficient for the development of cancer (2, 3, 22). Further acquired mutations, tissue expansion, local invasion, and distant metastasis are secondary events arising once this platform is established (2). We show herein that autocrine hGH activates a transcriptional program in human mammary carcinoma cells that is sufficient to provide such a platform, to release the mammary epithelial cell from normal proliferative arrest, and allow oncogenic transformation.

The essential role of functional synergy between proliferative and antiapoptotic signals at the early stage of mammary tumorigenesis has been recently highlighted by use of three-dimensional epithelial cell culture system (4, 27, 31). The mammary gland is composed of individual acinar units containing a hollow lumen and surrounded by polarized epithelial cells. The development and maintenance of this polarized structure is pivotal to the control of cell proliferation and cell survival required for the continuation of the differentiated state (4, 23, 27). The pathogenesis of mammary carcinoma requires the disruption of this intact, well-ordered architecture. Notably, filling of the luminal space is a hallmark of early epithelial tumors, such as atypical hyperplasia and ductal carcinoma *in situ* (4, 27, 31). Elegant studies from Debnath et al. (27) showed that only combined disruption of proliferative suppression (via Cyclin D1 overexpression) and luminal apoptosis (via Bcl-2 overexpression) resulted in luminal filling in human mammary acini. Autocrine hGH alone is sufficient to provide the platform for luminal filling due to transcriptional regulation of both *cyclin D1* and *Bcl-2*. Autocrine hGH production in the mammary epithelial cell is presumably a regulator of normal acinar development. We have observed the highest level of nonpathologic autocrine GH expression during pubertal development of the murine mammary gland.[6] Pathologic maintenance of autocrine *hGH* production after fulfillment of its normal developmental role would result in luminal filling and neoplastic progression of the human mammary gland. The observation that the *hGH* receptor has also been shown to be highly expressed in the human mammary stem cell population compared to differentiated mammary epithelial cells is supportive of this notion (32). The aberrant structures produced by *ex vivo* autocrine production of hGH are similar to *in vivo* ductal carcinoma *in situ* and indicative that autocrine hGH may possess a pivotal role in the early stage of mammary tumorigenesis.

Creation of a tumor cell from a normal cell requires both immortalization and oncogenic transformation (1). Primary rodent cells are easily transformed into tumor cells by two concomitantly introduced oncogenes. Only recently have human cells been oncogenically transformed by a triple 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-*ras*V12 (1). hTERT expression immortalizes human mammary epithelial cells. Immortalization is not sufficient to create an oncogenically transformed cell (1, 2). We have driven the oncogenic process in immortalized mammary epithelial cells by introduction of only the *hGH* gene under conditions in which the cell is maintained in a nontransformed state. Autocrine hGH evidently also requires the presence of as yet undefined cofactors for oncogenic transformation, as colony formation in soft agar requires serum and *in vivo* tumor formation was only achieved in the presence of Matrigel (this study). We also have evidence that autocrine hGH increases telomerase activity in human mammary carcinoma,[7] and this raises the intriguing possibility that enhanced expression of autocrine hGH may be sufficient to both immortalize and oncogenically transform the human mammary epithelial cell. However, the delineation between immortalization and oncogenic transformation as distinct processes may not be as clear as initially proposed (see ref. 33 for a review). This is evidenced by the observation that the overexpression of *hTERT* itself in the human mammary epithelial cell has been reported to cause the autocrine/paracrine secretion of multiple growth factors (34, 35) and, consequently, that simple *hTERT* overexpression by itself may contribute to tumor formation (36). In any case, autocrine hGH has been established as an important promoter of oncogenic transformation.

We note here that the oncogenic transforming effect of hGH is exclusive to autocrine-produced hGH and is not observed with exogenous administration of hGH. The lack of effect of exogenous hGH on oncogenicity is concordant with a large database of patients (37) in whom administration of exogenous hGH does not alter the relative risk for development of mammary carcinoma. Microarray analysis of 19,000 human genes has identified a subset of 305 genes in a human mammary carcinoma cell line that are exclusively regulated by autocrine hGH[8] although autocrine and exogenous hGH also regulated 167 common genes. It is therefore apparent that autocrine and exogenous hGH behave as distinct entities. Indeed, we have previously demonstrated that autocrine hGH, compared with exogenous hGH, differentially regulates the transcriptional activity of HOXA1, itself a powerful human mammary epithelial oncogene (19) and shown herein to be required for autocrine hGH–stimulated oncogenic transformation. Furthermore, we have also demonstated that autocrine hGH differentially regulates apoptosis in comparison to exogenous hGH in human mammary carcinoma cells (16) related to differential gene transcription of antiapoptotic (CHOP/GADD153; ref. 18) and proapoptotic (PTGF-β; ref. 38) factors. What remains to be clarified is the precise mechanism for the differential oncogenic effect of autocrine hGH, although the subset of exclusively autocrine hGH regulated genes will presumably contain many of the oncogenic determinants of autocrine hGH. The differential mode of presentation of autocrine hGH to the cell (low concentration but continuous secretion as opposed to high but transient concentrations of exogenous hGH, discussed in ref. 39) may contribute to differential gene expression; analogously different secretory patterns of pituitary growth hormone possess disparate effects (40). In any case, we have demonstrated that autocrine hGH is capable of stimulating oncogenic transformation of human mammary epithelial cells, whereas exogenous hGH is not. The small but significant inhibition of autocrine hGH–stimulated oncogenic transformation by exogenous hGH may simply be due to shift of the bell-shaped hGH dose-response curve. hGH has been proposed to initiate signal transduction by homodimerization of its receptor, with high concentrations of

[6] S. Mukina and P.E. Lobie, unpublished observations.

[7] Chen et al, submitted for publication.

[8] Xu et al., in revision.

hGH inhibitory, due to excess binding of the higher affinity site 1 to free receptor molecules (for a review, see ref. 26). Indeed, exogenous hGH does not significantly alter soft agar colony formation in MCF-7 vector cells in which no autocrine hGH is produced. Alternatively, exogenous hGH additional to autocrine hGH may interfere with receptor processing and/or availability or directly mitigate certain signal transduction processes stimulated by autocrine hGH.

Pathologic, environmental, nutritional, and pharmacologic factors that result in increased mammary epithelial hGH production will presumably contribute to neoplastic progression of the human mammary gland (12, 22, 41). In this regard, it is interesting that a known inhibitor of mammary carcinogenesis *in vivo*, vitamin D, also inhibits hGH gene transcription in a human mammary carcinoma cell line (42). Functional antagonism of hGH (12, 22) and the molecular pathways it uses (1, 2, 26), will therefore constitute novel adjunct therapeutic approaches to both the prevention and treatment of human mammary gland neoplasia.

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