Human Breast Cancer Progression Can Be Regulated by Dominant Trans-Acting Factors in Somatic Cell Hybridization Studies

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INTRODUCTION

Our contemporary thinking of the events involved in human BCP3 (such as tumor progression) in general centers largely on genetic changes that include mutations, translocations, amplifications of oncogenes, and the loss of heterozygosity of suppressor gene deletions (1). Therefore, both dominant-acting and recessive-unmasking genetic events contribute to breast cancer tumorigenesis, in which the central abnormality seems to involve a derangement in growth control. A good deal of progress has been made recently along these lines because perturbations of growth can be easily assayed in vitro (2). However, breast cancer tumorigenesis in vivo includes not only a derangement of growth but also a progression to an ER-negative, estrogen-independent, antiestrogen-resistant, EGFR-expressive, and highly metastatic phenotype (3). The molecular and biochemical mechanisms behind this progression are not well defined. Most studies of breast cancer have focused on one or another aspect of this progression but have not found a common pathway. By constructing stable and complete human-human somatic cell fusions between a highly metastatic, undifferentiated, ER-negative line of melanoma lineage and the estrogen-dependent, ER-positive MCF-7 line, this study produced hybrids that were ER negative, highly expressive of EGFR, estrogen independent, estrogen unresponsive, fully tumorigenic, and highly metastatic. ER negativity was on the basis of complete suppression of ER transcription as evidenced by Northern blot analysis and nuclear run-on assay, not on the basis of gene rearrangement. EGFR positivity was not due to gene amplification or rearrangement but rather to increased EGFR transcription. Mechanisms, including ras activation, fibroblast growth factor 4 expression, and human DNA methyltransferase activation causing ER promoter methylation, which are respectively known to induce estrogen-independent growth, induce spontaneous metastasis, and decrease ER levels in breast carcinoma experimentally, were not mechanisms operating in the hybrids. This model demonstrates that many of the common denominators of human breast carcinoma progression can be regulated by dominant trans-acting factors.

MATERIALS AND METHODS

Creation and Verification of Fusions

The study fused a highly metastatic and undifferentiated human line, C81614 (9), which exhibits a high rate of spontaneous metastasis in SCID and athymic (nude) mice, complete estrogen independence and unresponsiveness, antiestrogen resistance, and high EGFR expression, with the well-studied, nonmetastasizing, ER-positive, estrogen-dependent, EGFR-negative MCF-7 line, which exhibited estrogen-independent growth and tumorigenicity, estrogen unresponsiveness, ER suppression at the level of transcription, high EGFR expression, and high spontaneous metastasis. Because previous somatic cell hybridization studies between MCF-7 and an immortal mammary epithelial cell line exhibited features of the more “normal” parent, which included repression of the MCF-7 tumorigenic phenotype (8), our fusion results showing the progression of the MCF-7 phenotype to a more undifferentiated phenotype were intriguing. Our somatic cell hybridization studies demonstrate for the first time that many of the common denominators of human BCP can be regulated by trans-dominant factors.

Somatic Cell Hybridizations

Cells were fused as described previously (10), with modifications. Briefly, 1 × 10^5 cells of each parental line were mixed and plated into 60-mm culture dishes (Corning Glass, Corning, NY) with MEM + 10% FCS and were

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3 The abbreviations used are: BCP, breast cancer progression; ER, estrogen receptor; EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; FGF-4, the fourth member of the FGF family; FGF-4, the fourth member of the FGF family; NK1C, nullizygous mutant.

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incubated overnight at 37°C in 5% CO₂/95% air. In some fusions, unequal numbers of parental cells were mixed to optimize successful hybridization. After pretreatment of cells with DMSO or glycerol, 3 ml of 50% (w/v) polyethylene glycol (M₆ = 1000; Fluka, Bucha, Switzerland) were added onto the dish and were incubated at room temperature for 1–2 min. After removing polyethylene glycol, the cells were washed 3 times with basal MEM (no FCS) and were incubated in growth medium for 24 h at 37°C in 5% CO₂/95% air. The cells were then harvested with trypsin-EDTA (Sigma Chemical Co.), were suspended in MEM + 10% FCS, and were plated into 100-mm tissue culture dishes at 8 × 10⁶ cells in growth medium containing both 0.4–0.6 mg/ml G418 and 0.05–0.1 mg/ml hygromycin B. Emerging clones were identified within 3–4 weeks and were selectively removed with cloning rings.

**Flow Cytometric Analysis**

One × 10⁶ cells from each cell line to be analyzed were harvested in trypsin-EDTA, were spun to discard growth medium, were suspended in 1 ml of hypotonic staining buffer (0.1 mg/ml propidium iodide (Calbiochem), 0.3% Triton X-100, 20 µg/ml RNase A (Sigma Chemical Co.), and 1 mg/ml sodium citrate) and were analyzed on a FACScan (Becton Dickinson, Mountain View, CA; Ref. 11).

**Chromosomal Analysis**

Chromosomal analysis of the lines was carried out by Giemsa-banded staining (12). The mean, range, median, and modal chromosomal number were calculated from 20 chromosomal spreads. Based predominantly on the modal numbers and ranges as well as the presence of marker chromosomes of each of the parents and their fusion products, it was determined which clones produced by somatic cell fusion were complete and stable. By this analysis, clones exhibiting full and stable fusion were selected for subsequent study.

**Receptor Assays**

One × 10⁷–10⁸ cells (10–100 mg) were homogenized in TEGSM buffer (10 mM Tris, 1.5 mM EDTA, 10% glycerol, 1 mM sodium molybdate, and 1 mM monothioglycerol; pH 7.4). The cell suspension was centrifuged at 2000 rpm for 5 min at 4°C to bring down large particles and debris. The supernatant was then centrifuged at 100,000 × g for 30 min at 4°C. The high speed supernatant (cytosol) was removed and was used for the ER binding assay, and the crude high-speed membrane pellet was used for the EGFR assay. Protein content for cytosol (ER assay) and crude membrane preparation (EGFR assay) were measured by the method of Lowry et al. (13) with BSA standards (Sigma Chemical Co.). Mouse submaxillary gland GEF (Sigma Chemical Co.) iodinated with Na¹²⁵I by the chloramine-T method of Hunter and Greenwood (14) to a specific activity of 20–50 µCi/µg was used in the EGFR assay. Specific cpm were converted to binding in femtomoles as determined by the specific activity of the¹²⁵I-labeled GEF used (15). ERs were determined in tumor cell cytosolic fractions by a conventional multiple-dose dextran-coated charcoal assay (16, 17). ER and EGFR levels were also measured by established methods for immunocytochemical receptor analysis (18–20).

**Molecular Studies**

**Probe Preparations.** Plasmid DNA was isolated using the Qiagen Plasmid kit (Qiagen, Inc., Chatsworth, CA) according to manufacturer’s directions. Probes were prepared by linking fragments of interest from plasmids and gel, purifying them using an electroleuter (International Biotechnologies, Inc., New Haven, CT). Labeling was accomplished by random-prime labeling (Multiprime 228 DNA Labeling System, Amersham, Arlington Heights, IL) of 25 ng of probe with [α-³²P]dCTP (ICN Pharmaceuticals, Irvine, CA) at a specific activity of ≥5.0 × 10⁶ cpm/µg. Sephadex TEx Select-D G-50 columns (5 Prime-3 Prime, Inc., Boulder, CO) were used to remove unincorporated nucleotides. The HEO plasmid (21) containing the human ER cDNA (pGOR) was kindly provided by Dr. Pierre Chambon (Institut de Chimie Biologique, Strasbourg, France). The pOR3 ER cDNA was purchased from the American Type Culture Collection. The plasmid containing the EGFR cDNA probe (PE7; Ref. 22) was purchased from the American Type Culture Collection. The nm23 cDNA probe (23) was kindly provided by Dr. Patricia Steeg (NIH, Bethesda, MD). The human DNA methyltransferase cDNA probe was a gift from Dr. Paula Vertino (Johns Hopkins Oncology Center, Baltimore, MD; Ref. 24).

**DNA and RNA Extractions.** High molecular weight DNA was extracted as described (25) from confluent cultures by lysing cell monolayers with lysis buffer [10 mM EDTA, 10 mM Tris-HCl (pH 8.0), 0.5% SDS, and 200 µg/ml proteinase K] and incubating the lysate overnight at 55°C. After fractionating digested DNA on a 0.8% agarose gel, the gel was depurinated (26) by soaking in 0.25 M HCl for 15 min and rinsing in water. DNA from pretreated gel was transferred to positively charged nylon membrane in 0.4 M NaOH, then rinsed in 2 × SSC, and was UV cross-linked using 1200 µl in a Stratalinker (Stratagene, La Jolla, CA). Hybridizations were performed with QuikHyb solution (Stratagene). RNA was isolated by lysis of confluent cultures with Ultraspec™ Isolation System (Biotecx, Houston, TX), according to manufacturer’s directions. Polyadenylated mRNA (5–10 µg), isolated using an oligo-dexoxythymidylylate column (5 Prime-3 Prime, Inc.), was fractionated on a 1% agarose-formaldehyde gel and was transferred to positively charged nylon membranes (Boehringer Mannheim, Mannheim, Germany) in 20 × SSC and was UV cross-linked using 1200 µl in a Stratalinker. Hybridizations in QuikHyb and exposure to film were performed as in DNA analysis.

**Nuclear Run-on Assay.** Nuclear run-on assays were performed according to methods described previously (27).

**Cell Culture Studies**

**Hormone and Growth Factor Studies.** To assess the effects of 17-β-estradiol and EGF on cell growth, neo/hyg C816l, neo/hyg MCF-7, and the hybrid cells were grown in phenol red-free MEM + 10% charcoal/dextran-treated FCS (Hyclone Laboratories, Logan, UT) and 2 mM glutamine (Life Technologies, Inc.), with and without 10 mM 17-β-estradiol. The effect of EGF (Life Technologies, Inc.) on the hybrid cells was investigated by growing the cells in the previously mentioned media combinations with and without added EGF (10 ng/ml). The cells were plated in 100-mm tissue culture dishes, and cell counts were determined with a Coulter counter (Coulter Electronics, Marietta, GA).

**Methylation Inhibition Studies.** The hybrid cells were grown in regular media with and without added 5-azacytidine (2.5–5.0 µM) and/or 5-aza-2’-deoxycytidine (0.5–0.75 µM; Sigma Chemical Co.) for 10–14 days. Fresh drugs with media changes were added every fourth day. After this period of treatment, cells were harvested and subjected to ER transcript analysis by Northern blot and nuclear run-on assay, and ER protein analysis.

**Tumorigenicity and Metastasis Studies**

**Animal Injection Studies.** Ovariectomized 4–6-week-old female nude mice (nu/nu mutants on a BALB/c background) and 4–6-week-old male SCID mice were used. One × 10⁷–10⁸ cells suspended in 0.2 ml basal MEM (no FCS) were injected s.c. in the right dorsolateral flank region with and without added 17-β-estradiol (0.72 mg/pellet with biodegradable carrier binder; Innovative Research of America, Sarasota, FL), and tumors were allowed to grow for up to 15 weeks or until the animals became moribund. Metastases were detected by histological analysis of the lungs and by retrieval of pulmonary colonies through collagenase digestion, antibiotic selection, and clonal outgrowth. Metastatic clones retrieved in this manner were subjected to all of the previously mentioned flow cytometric and chromosomal studies to verify their karyotypic stability.

**Natural Killer Cell and Macrophage Cytotoxicity.** Recovered primary and metastatic clones were tested for sensitivity to macrophage and natural killer cell cytolsis, according to established methods (28–30).

**General Experimental Design and Statistical Analysis.** All experiments were performed with groups of 10 mice. Results were analyzed with standard tests of statistical significance, including a two-tailed Student’s t test and a one-way ANOVA.

**RESULTS**

Somatic cell fusions carried out between C816l and MCF-7 uniformly exhibited the undifferentiated phenotype of C816l. In these human-human hybrids, because the results were counter to those of previous studies in which the more malignant properties were sup-
pressed in fusions with less malignant cells (8), the claim that our hybrids represented true and complete fusions and not improperly fused subsets of C8161 was intensely verified by chromosomal marker studies, polymorphic genetic loci, DNA ploidy studies, and control fusions. Under the double antibiotic selection conditions applied to each parent alone, a log kill $\geq 1 \times 10^6$ was achieved, eliminating all traces of each parent. No clones were therefore observed on control plates from self-fusions, e.g., hygC8161 × hygC8161 or neoMCF-7 × neoMCF-7, or vice versa. With the C8161 × MCF-7 fusions, 30 clones initially emerged. Of these, approximately 60% either ceased dividing or were not recovered after trypsinization. Another 20% were incompletely fused. Five clones eventually emerged from each fusion that were completely and stably fused on the basis of DNA ploidy and karyotype analysis (Figs. 1 and 2; Table 1). Hence, the final cell hybrids were generated at a frequency of $5 \times 10^{-6}$. The hybrids were morphologically different from both antibiotic-transfected parents. In addition, the hybrid clones were demonstrated to contain polymorphic loci from each parent as discussed below. The hybrid clones remained stable and retained 95–100% of their chromosomal complement through subsequent in vitro and animal passages. Self-fusions (neoC8161 × hygC8161 and neoMCF-7 × hygMCF-7) showed no alterations in phenotypic traits when compared to their respective parents. Therefore, on the basis of all of these findings, the apparent dominance of the C8161 phenotype in the fusions was not an artifact of an improperly fused subset of C8161 but was rather a reflection of true genetic dominance.

ER and EGFR immunocytochemical studies indicated high membrane EGFR and no nuclear ER immunoreactivity in C8161, high nuclear ER and no membrane EGFR immunoreactivity in MCF-7, and high membrane EGFR and no nuclear ER immunoreactivity in the C8161 × MCF-7 hybrids (Fig. 2). The receptor immunocytochemical studies were substantiated by quantitative ligand binding receptor assays. C8161 exhibited EGFR levels of $118$ fmol/mg membrane protein and no detectable levels of ER; MCF-7 exhibited no detectable levels of EGFR and $60$ fmol/mg cytosolic protein of ER; the C8161 × MCF-7 hybrids, like C8161, exhibited high EGFR levels ranging from $110–130$ fmol/mg membrane protein and no detectable ER (Fig. 3). The inverse relationship between EGFR and ER expression was observed in all of the hybrid clones.

To investigate the possible mechanisms of this differential expression of EGFR and ER, a number of molecular studies were carried out. Comparative Southern blots of C8161, MCF-7 parents, and C8161 × MCF-7 hybrid clones probed with EGFR and ER cDNAs revealed no evidence of gene amplification or rearrangement as a mechanism of altered expression (Fig. 4). Karyotype analysis revealed the C8161 line to contain two normal appearing chromosome number 7s at the location of the EGFR gene. The MCF-7 line contained a known ER PvuII polymorphism (31) that could be used to mark the presence of the MCF-7 gene in the hybrid clones. Comparative Northern blots of C8161, MCF-7, and C8161 × MCF-7 hybrid clones probed with EGFR and ER confirmed the ligand binding and immunocytochemical receptor data (Fig. 4). Prominent 10- and 5.6-kb EGFR transcripts were detected in the C8161 and hybrid clones but were completely absent in MCF-7, and these findings were confirmed by a nuclear run-on assay showing that EGFR transcription in the hybrid resembled that in the C8161 line (Fig. 4). With respect to ER expression, a prominent 6.3 Kb ER transcript was present in MCF-7 but was completely absent in C8161 and the hybrid clones. Reverse transcription-PCR using first-splice-site ER primers verified the absence of any ER transcripts in the hybrid clones (data not shown). An ER nuclear run-on assay confirmed the complete absence of ER transcription in the hybrid clones (Fig. 4). Because complete absence of transcription was observed in all five fusions, all of which apparently contained the ER gene intact without evidence of deletions or rearrangements, it was considered highly unlikely that mutations within cis elements of the ER promoter were responsible for absence of ER transcription in the hybrid clones; rather, the complete absence of ER transcription in the hybrids supported a mechanism involving dominant trans-acting factors. Because the presence of increased transcription of EGFR also did not involve gene amplification or rearrangement, these findings also supported a mechanism involving dominant trans-acting factors as well. Because recent studies had suggested that increased methylation of the CpG island in the 5’ promoter of the ER gene correlated with silencing of expression and that this effect was mediated by increased levels of DNA methyltransferase in ER-negative human breast carcinoma cell lines (24, 32—35), we examined possible cis and trans methylation differences among the C8161, MCF-7, and C8161 × MCF-7 hybrid clones. No differences in steady-state levels of DNA methyltransferase were appreciably between C8161 and MCF-7, and in fact, steady-state levels were reduced (by up to 65%) in the C8161 × MCF-7 hybrid clones (Fig. 5). Southern blots of the ER promoter region and internal ER gene sequences hybridized with a probe (pOR8) spanning a large ER region revealed no differences in methylation patterns among C8161, MCF-7, and C8161 × MCF-7 hybrid clones. Southern blots hybridized with a short 0.3-kb PvuII fragment of the pOR3 cDNA specifically designed to examine the CpG island in the 5’ ER promoter region revealed no evidence of methylation in this island (Fig. 5). The banding pattern present in the hybrid clones was that exhibited by

Fig. 1. The appearance of one emerging C8161 × MCF-7 hybrid clone is depicted by phase-contrast microscopy. Note the scattered heterokaryons among the predominant hybrid cells exhibiting a single nucleus (A). The completely fused and stable nature of this hybrid was confirmed by chromosome analysis. Metaphase spreads from this hybrid clone revealed a modal chromosome number of 149 (B).
ER-positive breast carcinoma cell lines rather than that exhibited by ER-negative breast carcinoma cell lines. This finding was in strong contrast to the fact that the hybrid clones were strongly ER negative. As further proof that methylation of the 5' promoter region was not mediating the effects on ER expression noted in the hybrid clones, cultured hybrid cells were treated with two inhibitors of DNA methylation, 5-azacytidine and 5-aza-2'-deoxycytidine, for 14 days, and ER levels were examined by Northern blot, nuclear run-on, ligand binding receptor, and immunocytochemical receptor assays. No ER mRNA or ER protein could be detected with these experimental manipulations. This is in contrast to the effects of these agents in restoring ER expression in select ER-negative breast carcinoma lines shown in recent studies (36). Our studies further support the presence of trans-dominant factors that suppress ER expression in our hybrids. In general, there has been a paucity of studies to date dealing with down-regulation of ER at the level of transcription and, to date, no silencing trans-acting factors have been identified (37).

Next we examined the effects of estrogen and EGF on the growth of the antibiotic gene-transfected parental cell lines as well as the hybrid clones. Both C8161 and the hybrids were completely estrogen independent, whereas the MCF-7 line was completely estrogen dependent for its growth in monolayer culture (Fig. 6). Interestingly, the growth rates of both C8161 (with or without estrogen) and MCF-7 (with estrogen) were significantly greater than that observed with the hybrid clones. The doubling time of the C8161 line was approximately 12 h; the MCF-7 doubling time was approximately 24 h; and
Tumorigenicity and spontaneous metastasis of the C8161, MCF-7, C8161 + MCF-7, and C8161 × MCF-7 hybrid clones. Results depict the number of positive/10 mice. Whereas the tumorigenicity of both C8161 and the hybrids was completely independent and unresponsive to the effects of estrogen, the tumorigenicity of MCF-7 was strictly estrogen dependent (P < 0.001). Both C8161 and the hybrid clones had a very high incidence of spontaneous metastasis, whereas the MCF-7 line, even in the presence of estrogen, did not metastasize (P < 0.001). C8161 exerted no effect on MCF-7 when the cells were mixed at varying ratios. The same effects were observed with neo C8161/hyg MCF-7 experiments as with hyg C8161/neo MCF-7 experiments.

Table 1 Characteristics of the C8161 × MCF-7 hybrids

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Chromosomes</th>
<th>Tumorigenicity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Metastasis&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
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<td>Parents</td>
<td></td>
<td>+E, −E</td>
<td>+E, −E</td>
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<tr>
<td>neo/hyg C8161 + MCF-7</td>
<td>82 (69-95)</td>
<td>10</td>
<td>10</td>
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<tr>
<td>neo/hyg MCF-7</td>
<td>75 (65-78)</td>
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<td>0</td>
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<tr>
<td>neo/hyg C8161 + MCF-7</td>
<td>75 (65-78)</td>
<td>5</td>
<td>0</td>
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<tr>
<td>Hybrid clones</td>
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</tr>
<tr>
<td>1</td>
<td>141 (102-152)</td>
<td>9</td>
<td>10</td>
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<td>149 (139-155)</td>
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<td>141 (105-160)</td>
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<td>5</td>
<td>157 (139-172)</td>
<td>10</td>
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<sup>a</sup> Tumorigenicity in the presence and absence of supplemental estrogen in ovariectomized female nude mice. E, estrogen.

<sup>b</sup> Spontaneous metastasis in the presence and absence of supplemental estrogen in male SCID mice.

<sup>c</sup> With the C8161 + MCF-7 co-injection experiments, the latency, percentage of tumorigenicity, and growth of C8161 were identical to the C8161-alone injections. The data displayed depict the recovered MCF-7 cells when varying ratios of C8161 and MCF-7 cells were mixed together in the presence and absence of supplemental estrogen. For example, varying ratios of hyg C8161 and neo MCF-7 were mixed together including 10:1, 1:1, and 1:10. Five × 10<sup>5</sup> total cells were injected into the flank with and without supplemental estrogen. After the primary tumors emerged (4–6 weeks) and reached 1.5 cm in size, they and the lungs were extirpated and subjected to collagenase digestion and culturing of the liberated cells in the appropriate antibiotic selective media. The only metastatic colonies retrieved were in hygromycin B selective media (i.e., hyg C8161); no neo MCF-7 colonies were retrieved.

Fig. 3. The pattern of ER and EGFR immunoreactivity was confirmed with conventional ligand binding receptor assays. The levels of ER and EGFR in C8161, MCF-7, and a typical hybrid C8161 × MCF-7 clone are depicted. The inverse correlation between ER and EGFR held in all these cell lines.

Tumorigenicity and spontaneous metastasis of neo/hyg C8161, neo/hyg MCF-7, C8161 + MCF-7 co-injections, and C8161 × MCF-7 hybrid clones were compared (Table 1). Whereas the tumorigenicity of both C8161 and the hybrid clones was completely independent and unresponsive to the effects of estrogen, the tumorigenicity of MCF-7 was strictly estrogen dependent. Although the antibiotic-resistant MCF-7 cells had been reported to have a higher degree of estrogen-independent tumorigenicity than their untransfected parents in previous studies (38), the antibiotic-resistant clones of MCF-7 that were used in our study did not show any tumorigenicity in the absence of estrogen. Even in the presence of estrogen, the MCF-7 tumors that arose were slow-growing compared to C8161. Both the C8161 and hybrid clones had a very high incidence of spontaneous metastasis (Table 1, Fig. 7), whereas the MCF-7 line, even in the presence of estrogen, did not metastasize (Table 1). There were no significant alterations in susceptibility to either natural killer cell or macrophage cytolyis (stimulated or unstimulated) among the C8161, MCF-7, or hybrid clones. All of the hybrid clones exhibited relatively low susceptibility to natural killer cell and macrophage cytolyis.

Because of previous studies implicating nm23 (39, 40), p53 (3, 41), mutated ras (38), neu (3), and FGF-4 (42, 43) in human and experimental BCP (including the progression to hormonal independence), we undertook studies in C8161, MCF-7, and the hybrid clones designed to investigate whether any of these genes might be involved. No differences in steady-state nm23 levels among C8161, MCF-7, or the C8161 × MCF-7 hybrid clones could be appreciated by Northern blot analysis (Fig. 5). p53 showed no alterations by single-strand conformation polymorphism analysis, and altered p53 could not be detected by immunocytochemical studies; N-ras and H-ras showed no evidence of point mutations; neu was not amplified by Southern blot analysis; and FGF-4 transcripts could not be detected by Northern blot analysis (data not shown). Furthermore, in co-injection experiments mixing C8161 cells with MCF-7 cells in varying proportions, the C8161 cells grew as they normally did when injected alone. Hence, there was no evidence that MCF-7 cells either stimulated or suppressed the growth of C8161. More importantly, C8161 did not stimulate the growth, tumorigenicity, hormonal independence, or metastasis of MCF-7 because no MCF-7 cells could be recovered from co-injection tumors that arose in the absence of estrogen (Table 1). Hence, secreted paracrine growth factors such as FGF-4, which could have been produced by C8161 and stimulated the growth of MCF-7 cells, were not in evidence. Hence such secreted growth factors, working in an autocrine manner, were probably not the mechanism of the C8161 × MCF-7 hybrids' acquisition of estrogen-independent growth and metastasis.

DISCUSSION

Although the concept of breast cancer tumor progression involving a series of genetic events has evolved relatively recently, it has been known for decades that a certain percentage of human breast cancers...
initially respond to hormonal therapy and later become refractory. It was not until the ER was identified in ligand binding assays that one could predict, in part, which tumors were likely to respond initially to hormonal therapy and which were not (45, 46). Because the ER is a trans-activating transcriptional factor when bound to its ligand, most of the research on ER has focused on the identification of the genes that it regulates. Its regulation of such genes facilitated considerably when the ER gene was cloned (44). The vast majority of human breast cancers show no structural alteration of the ER gene, and early studies did not demonstrate significant amplifications, rearrangements, deletions, or altered methylation to explain why certain breast cancers are ER positive and why some are not (45, 46). Our ability to address the molecular mechanism of ER action was facilitated considerably when the ER gene was cloned (44). The vast majority of human breast cancers show no structural alteration of the ER gene, and early studies did not demonstrate significant amplifications, rearrangements, deletions, or altered methylation to explain why certain breast cancers are ER positive and why some are not (45, 46). Because the ER is a trans-activating transcriptional factor when bound to its ligand, most of the research on ER has focused on the identification of the genes that it regulates. Its regulation of such genes

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**Fig. 4.** Southern blot of C8161, MCF-7, and a typical C8161 × MCF-7 hybrid clone probed with EGFR and ER probes. A, 20 μg of genomic DNA were digested with 60 units of EcoRI (New England Biolabs, Beverly, MA) for 16 h, were fractionated on a 0.8% agarose gel, were blotted to a nylon membrane, and were hybridized to a 2.4-kb 32P-labeled Cdi fragment of plasmid containing PE7 EGFR cDNA. Characteristic bands of 9.4, 6.5, 4.3, 3.6, and 2.1 kb appeared. The banding pattern was identical in C8161, MCF-7, and C8161 × MCF-7 fusion. No obvious gene amplifications or rearrangements of EGFR were apparent. B and C, 20 μg of genomic DNA were digested with (B) 50 units of EcoRI or (C) 50 units of PvuII (Life Technologies, Inc.) for 16 h, were blotted to a nylon membrane, and were hybridized to a 1.8-kb 32P-labeled EcoRI fragment of HEO plasmid containing human ER cDNA (pOR8). With EcoRI digestion and hybridization to pOR8, characteristic bands of 8.8, 7.8, 7.0, 4.6, 3.5, 2.9, 2.3, and 1.5 kb appeared. No differences were appreciated between the cell lines. With PvuII digestion (C), characteristic bands of 13, 6.8, 5.6, 3.9, and 1.2 were apparent. Note the appearance of an extra 1.5-kb band (arrow) in MCF-7 and the C8161 × MCF-7 hybrid, indicative of a known PvuII ER polymorphism. No rearrangements of the ER gene were apparent in any of the cell lines. Northern blot of C8161, MCF-7, and a typical C8161 × MCF-7 hybrid probed with EGFR (D) and ER (E) cDNAs are shown. Northern blots were prepared by running 5–10 μg polyadenylated mRNA on each 1% agarose-formaldehyde gel, transferring RNA to a nylon membrane, and hybridizing with the 2.4-kb EGFR probe and the 1.8-kb ER probe used previously in Southern blot analysis. Prominent 10- and 5.6-kb EGFR transcripts were present in C8161 and the hybrids but were absent in MCF-7 (D). A prominent 6.3-kb ER transcript was present in MCF-7 but was completely absent in C8161 and the hybrids (E). Reverse transcription PCR confirmed these findings. The nuclear run-on assay (F) demonstrates: no active ER transcription in C8161 or the hybrids; in contrast, EGFR was actively transcribed in the hybrids; β-actin was used as a positive transcriptional control.

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**Fig. 5.** Northern blots of MCF-7, C8161, and a typical C8161 × MCF-7 hybrid probed with DNA methyltransferase and nm23 cDNA. Using a 2.5-kb EcoRI fragment of human DNA methyltransferase cDNA and a 0.9-kb BamHI fragment of nm23 cDNA as probes, the hybrid showed less steady-state 5.3-kb methyltransferase transcript levels (top) and approximately equal steady-state 0.8-kb nm23 transcript levels (middle) compared to its C8161 and MCF-7 parents. Normalization was with β-actin (bottom). Southern blot demonstration of potential cis- and trans-methylation differences affecting ER transcription in the C8161 × MCF-7 hybrids is shown (B and C). B, Southern blot of ER promoter region of the hybrid probed with a 0.3-kb PvuII fragment of pOR3 ER cDNA; EcoRI digest, lane 1; EcoRI/HindIII digest, lane 2. The examined 5' region of the promoter (which included the 5'CpG island) revealed no detectable methylation and exhibited the characteristic 1.9- and 1.2-kb bands with EcoRI/HindIII digestion. The lack of methylation resembled the pattern seen in select ER-positive breast cancer lines and was in contrast to the methylated pattern reported in select ER-negative lines (32). C, Southern blot of the same ER promoter region of the C8161 × MCF-7 hybrid probed with the 0.3-kb PvuII fragment of pOR3 cDNA as in (B), but DNA digested with EcoRI (Lane 1), EcoRI/HindIII (Lane 2), and EcoRI/MspI (Lane 3) produced the same characteristic 0.3- and 0.2-kb bands with both EcoRI/HindIII and EcoRI/MspI digestion. Again, this unmethylated pattern was characteristic of ER-positive breast carcinoma cell lines.

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as the progesterone receptor, pS2, and cathepsin D has been studied intensely (47, 48). Because ER both binds its ligand and regulates transcription, much research has focused on the identification of the specific molecular domains that mediate these activities (49). Predictably, there has been an identification of mutant forms of ER that either lack the ability to bind ligand or the ability to activate transcription. These mutants have been observed in both cell lines and clinical specimens of human breast cancer (50—53). Some of these mutants are dominant negative (54) and bind both ligand and DNA but do not activate transcription, rather they interfere with the wild-type ER to bind to its cognate response element and activate transcription. Other mutants are dominant positive (55) and lack the ability to bind their ligand but nevertheless retain their ability to activate estrogen-responsive genes such as the progesterone receptor. However, these mutant forms of ER probably comprise a very small percentage of ER-negative or estrogen-independent clinical tumors. Although there is a strong correlation between ER protein levels and steady-state mRNA levels in human breast cancer (56), and although ER-negative cell lines and cases of human breast cancer usually exhibit an absence of ER transcripts, there have been comparatively few studies addressing the transcriptional regulation of ER until recently. In a study by Weigel and deConinck (4), there was no ER transcription in the ER-negative MDA-MB-231 line. Whether this was regulated by mutations in cis elements in the promoter or by trans-acting factors could not be determined. Because the progression of human breast cancer is characterized by diverse phenotypic changes and the altered expression of a number of different genes, we wondered whether these many changes could be induced pleiotropically, and we had the idea of effecting induction via somatic cell fusion. We chose the C8161 line instead of one of the more aggressive, ER-negative, high-EGFR-expressing breast carcinoma lines because our primary aim was to investigate whether the changes that characterize the progression of human breast cancer could be induced by somatic cell fusion, and we wanted the most aggressive and undifferentiated line available that we could use for this purpose. The C8161 line metastasizes early and very aggressively by 4—6 weeks after s.c. injection when it has reached a size of only 1.5 cm. At this stage, widespread metastases are both microscopically and grossly visible. In earlier experiments with this line, our laboratory had demonstrated that in somatic cell fusions between C8161 and nonmetastatic melanoma lines, the aggressive phenotype of the C8161 line dominated. Hence, based on these earlier experiments, we had reason to believe that C8161 might possess the necessary dominant trans-acting factors that could effect the changes in gene expression characteristic of human BCP in a recipient breast carcinoma line. Previous studies, in contrast, had demonstrated that even the most aggressive ER-negative breast carcinoma lines exhibit a comparatively low incidence of metastasis, requiring a much longer time-period of growth (i.e., months) to be detected. Other studies with breast carcinoma fusions had demonstrated that the phenotype of the more differentiated line predominates, presumably due to one or more dominant tumor suppressor factors. For these reasons, we felt that using C8161 as an experimental line to test our hypothesis of the existence of dominant trans-acting factors that can regulate human BCP made the most sense. In our study, because a complete absence of ER transcription was observed in all five fusions (all of which contained copies of the MCF-7 ER gene), it was considered highly unlikely that in every fusion, mutations of cis elements within the ER promoter were responsible for the absence of transcription. It was also demonstrated in our studies that DNA methyltransferase-mediated methylation of the CpG island in the 5' promoter region of the ER gene did not occur and hence could not have mediated the silencing of ER expression (Fig. 5). In our C8161 × MCF-7 hybrids, ER transcription was completely inhibited, despite the ER promoter's unmethylated status facilitating transcription. Predictably, treating our hybrid clones with inhibitors of DNA methylation was not effective in restoring ER expression. Our data then support a novel mechanism.

Fig. 6. Growth response to 17-ß-estradiol and EGF. Growth curve of one typical C8161 × MCF-7 hybrid clone in phenol red-free media with dextran/charcoal-treated FCS, ± 10 nm 17-ß-estradiol and ± 10 ng/ml EGF. The hybrid clone demonstrated both estrogen-independent growth and estrogen-unresponsive growth. No effect of EGF was appreciated.

Fig. 7. Gross (A) and microscopic (B) appearance of metastases of the C8161 × MCF-7 hybrids. The metastatic colonies could be retrieved from the lungs by collagenase digestion, antibiotic selection, and clonal outgrowth and could be subsequently studied.
independent of methylation involving dominant trans-acting factors. The data in our study showing no methylation effects on the ER promoter could perhaps be reconciled with recent studies (32, 36), which show a role for methylation in the ER promoter in the progression to ER negativity, by arguing that our model reflects very late and perhaps irreversible changes that are associated not only with ER silencing but with the acquisition of the estrogen-independent, estrogen-unresponsive, EGFR-positive, highly metastatic phenotype. Recent studies (32, 36) convincingly showing a role for DNA methylation in the reversible progression to ER negativity but not in the acquisition of EGFR positivity or the metastatic phenotype more likely reflect earlier stages of progression.

There has been a paucity of studies dealing with the transcriptional regulation of ER by DNA binding proteins. In a recent study, deConinck et al. (57) identified a factor, ERF-1, expressed in ER-positive breast carcinomas that binds to an important transcriptional regulatory element of the ER gene and augments expression (57). In another study, 12-O-tetradecanoylphorbol-13-acetate (37) was found to down-regulate ER, but the exact transcriptional mechanism was not addressed. Because 12-O-tetradecanoylphorbol-13-acetate activates the protein kinase C-dependent signal transduction pathway that results in the activation of AP-1, it was postulated that the mechanism of ER down-regulation could involve AP-1. However, because there have been no AP-1 sites identified in the human ER promoter, and because AP-1 usually stimulates rather than down-regulates expression, the story cannot be that direct and simple. Although analyses of both a rainbow trout and a human ER promoter (58, 59) have identified both putative enhancer and silencer motifs, a direct demonstration of cis/trans interactions silencing ER expression has not yet been made. To date, there have been no trans-acting factors identified that directly suppress expression of the human ER, despite the fact that these factors may play key roles in the progression of human breast cancer to the ER-negative phenotype. Although the absence of ERF-1 expression could account for decreased or even absent ER expression in our somatic cell hybrids, it is also possible that the hybrids contain trans-acting factors that can directly silence ER expression even in the presence of ERF-1.

The phenotypic loss of ER at whatever level it occurs is clearly not the phenotypic equivalent of the acquisition of hormonal independence. Although it could be argued that dominant-positive ER mutants provide one link between the ER and the acquisition of hormonal independence, this mechanism probably accounts for only a few cases clinically. In the majority of cases of human breast cancer, there is probably a dissociation of the growth response from the receptor, despite the presence of receptor (60). Although this may be mediated at least theoretically by altered hormone-response elements in growth factor genes or proto-oncogene mutations, these mechanisms remain speculative. The differences between ER expression and the acquisition of estrogen independence were recently illustrated in a study involving transfection of an ER cDNA construct into the ER-negative MDA-MB-231 line (61). In this study, estradiol actually inhibited the growth and hematogenous metastasis of the transfected line, whereas estradiol normally stimulates the growth of ER-positive breast cancer cell lines. This study indicated that factors other than ER were involved in the progression of breast cancer toward hormonal independence. Most studies dealing with the acquisition of hormonal independence have exploited growing cells in both the short-term and long-term absence of estrogen and obtaining clones showing estrogen independence (62, 63). However, the molecular mechanisms behind the emergence of these clones remain unknown. In this discussion, the difference between estrogen independence and estrogen unresponsiveness should be emphasized. Many of these estrogen-independent cell line variants retain their ability to be at least partially stimulated by estrogen and inhibited by antiestrogens. It should be emphasized that
regulating the metastatic phenotype in our hybrids were overriding the influence of nm23. Similarly, no role for altered p53 or amplified neu in our hybrids could be demonstrated. It should be emphasized that the most intensely studied estrogen-positive human breast cancer lines, including MCF-7 and T47D, are either nonmetastatic or, at best, weakly metastatic in athymic or SCID mice. Therefore, experimental manipulations that induce their metastatic behavior may provide insights into events that regulate the metastatic phenotype in vivo. Our somatic cell fusions of C8161 with MCF-7 exhibited a dramatic metastatic phenotype (Table 1; Fig. 7). Recently, the transfection of FGF-4 into MCF-7 cells induced a metastatic phenotype (42). The FGF-4–transfected clones were not only metastatic but also stimulated tumorigenicity of untransfected MCF-7 cells when co-injected into ovariectomized mice in the complete absence of estrogen supplementation. The transfectants, however, remained responsive to estrogen and presumably still expressed ER. Although members of the fibroblast growth factor family, including FGF-4, have been implicated in the tumorigenesis and metastasis of mouse mammary tumors (43), a direct role for them in human BCP and metastasis has, to date, not been demonstrated. In the C8161 × MCF-7 hybrid clones, FGF-4 transcripts were not detected; furthermore, in co-injection experiments, no effect of C8161 on MCF-7 growth, tumorigenicity, hormonal independence, or metastasis could be detected (Table 1), suggesting that the effects seen with somatic cell hybridization were not due to the secretion of either an autocrine or paracrine growth factor like FGF-4.

Most studies with somatic cell hybrids observe that the most differentially phenotyped parent of the hybrids predominates in the fusions. For example, in hybrids produced by somatic cell fusions of MCF-7 with a normal immortalized human mammary epithelial line, features of the more “normal” parent (which included loss of tumorigenicity, increased extracellular matrix gene expression, and regression of the MCF-7 phenotype) predominated, presumably due to one or more dominant tumor suppressor factors (8). Although it might have been anticipated then that our hybrids would also have shown features of the “more normal parent” (which would include the absence of metastasis, high ER expression, and significant estrogen responsiveness and dependence), our hybrids demonstrated exactly the opposite, showing progression of the MCF-7 phenotype to a more “malignant” phenotype. This hybrid model demonstrates that trans-dominant elements can induce this progression. Our studies offer somatic cell hybridizations between a highly undifferentiated melanoma line exhibiting markers of epithelial and adenoscarcinomatous differentiation and the MCF-7 line as a model to dissect those factors responsible for suppressing the ER-positive phenotype and those responsible for maintaining or inducing the EGFR-positive phenotype, estrogen independence, estrogen unresponsiveness, and spontaneous metastasis. We wish to point out, however, that our model does not yet provide evidence that the trans-dominant elements demonstrated are actually present in breast cancer cells that have undergone malignant progression. In many respects, our model may therefore have more to say about the relationship between EGFR and ER expression than human BCP per se. On the other hand, we feel that because ER expression and EGFR expression play a central role in human BCP, by addressing the regulation of ER and EGFR in our model, we may also in fact be addressing an issue relevant to human breast cancer. Our present study provides evidence that many of the common determinants of human BCP can be regulated by dominant trans-acting factors. Whether these factors and their mechanism of trans-dominance play a role in human breast cancer remains to be demonstrated.

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