Fibroblast Growth Factor 4 Transfection of MCF-7 Cells Produces Cell Lines That Are Tumorigenic and Metastatic in Ovariectomized or Tamoxifen-treated Athymic Nude Mice

Sandra W. McLeskey, Junichi Kurebayashi, Susan F. Honig, James Zwiebel, Marc E. Lippman, Robert B. Dickson, and Francis G. Kern


ABSTRACT

Successful antiestrogen treatment in patients with tamoxifen-responsive breast tumors is often followed by an outgrowth of tumors cells that are estrogen-resistant, implying that estrogen-dependent tumors can become estrogen-independent. In an effort to mimic this progression, we have transfected fibroblast growth factor 4 into MCF-7 cells, a human breast carcinoma cell line that is estrogen-dependent for growth in nude mice. This transfection results in cell lines that form progressively growing, metastatic tumors when injected s.c. into untreated or tamoxifen-treated ovariectomized nude mice. In contrast to the parental cell line, growth of transfected cells in ovariectomized nude mice is stimulated by tamoxifen treatment and inhibited by estrogen treatment of the mice. Parental MCF-7 cells were transfected with an expression vector for β-galactosidase, conferring the ability to convert the chromogenic substrate, 5-bromo-4-chloro-3-indoyl-β-galactoside, to a blue color and allowing the detection of their presence within tumors developing after coinoculation with fibroblast growth factor 4-transfected cells. The fibroblast growth factor 4-transfected cells could support growth and metastasis of the β-galactosidase-expressing parental cell line when both lines were coinjected into the same site in untreated or tamoxifen-treated, ovariectomized mice. These data suggest a possible role for fibroblast growth factors in the progression of breast tumors to an estrogen-independent, antiestrogen-resistant, metastatic phenotype. They also support a role for paracrine factors in mixed populations of tumor cells of differing states of malignant progression.

INTRODUCTION

A subset of estrogen receptor-positive human breast tumors is growth-stimulated by estrogen and is amenable to treatment with antiestrogens. The growth-stimulatory effect of estrogen on these tumors may involve autocrine and paracrine effects resulting from the induction of growth factors, as suggested by the finding that conditioned medium from estrogen-treated MCF-7 cells partially supports tumor growth in ovariectomized nude mice (1). Extension of this hypothesis suggests that estrogen treatment might interfere with the production of estrogen-induced growth factors, removing mitogenic stimulation of the tumor or perturbing the dynamic interaction between tumor and stroma. However, after a period of successful treatment, antiestrogen-responsive breast tumors may become refractory to antiestrogens. These estrogen-independent cells might have acquired the ability to constitutively express the same or related growth factors previously induced by estrogen, to influence stromal elements of the tumor or host immune function in a way more permissive of tumor growth, or to bypass external growth factor pathways completely. If the mechanism(s) underlying the progression to an estrogen-independent, metastatic phenotype were known, strategies for therapy of these currently untreatable malignancies could be developed.

FGFs are a seven-member family of proteins that are mitogenic for cells of mesodermal and neuroectodermal origin and are thought to be important in development and angiogenesis. FGFs have been shown to be transforming in NIH-3T3 cells (2) and have been implicated in tumorigenesis (3) and metastasis (4) of mouse mammary tumors. FGF-4 overexpression confers a tumorigenic phenotype on a human adenocarcinoma cell line, indicating that FGFs may also be important in the transformation of epithelial cells (5, 6). Because of the possible role of FGFs in neovascularization, tumorigenesis, and metastasis and the association of increased vascularity of breast tumors with a poorer prognosis (7, 8), we have transfected MCF-7 cells to test the ability of one secreted FGF family member, FGF-4, to convert a breast carcinoma cell line from one that is estrogen-dependent for in vivo growth to one that no longer requires supplemental estrogen for tumorigenicity. This transfection results in cell lines capable of forming progressively growing, metastatic tumors in ovariectomized and tamoxifen-treated nude mice. These transfected cell lines, which secrete FGF-4, are able to support the growth of the parental cell line when both cell types are injected into the same site under circumstances where the parental cell line is normally nontumorigenic. Moreover, parental cells were detected in metastases of these mixed tumors, indicating that coinjection of parental and FGF-4-transfected cell lines facilitates metastasis by the parental cells.

MATERIALS AND METHODS

Cell Lines. MCF-7 cells (passage 50) were maintained in a 37°C, 5% CO2 incubator in IMEM supplemented with 10% FBS. SW-13 adrenal carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in IMEM supplemented with 5% FBS. ML-20 cells were obtained as described (9) by transfecting MCF-7 cells (passage 50) with the pCHβ-Gal expression vector encoding the bacterial lacZ gene and subsequently establishing a clonal cell line with high levels of β-galactosidase expression.

Construction of pCNCKSEBl and Transfection of MCF-7 Cells. Control and fgg-4-containing eukaryotic expression vectors were constructed as described (5). MCF-7 cells were transfected by the method of Chen and Okayama (10) and G418-resistant clonal cell lines were established. RNA was isolated by the vanadyl ribonuclease complex method (11) and screened for fgg-4 mRNA by Northern analysis (12). Clonal cell lines with high levels of fgg-4 mRNA production (MKS-1, MKS-13, and MKS-15) were identified.

RNase Protection Assay. Thirty μg total RNA from each cell line were hybridized with 32P-labeled riboprobes for glyceraldehyde 3-phosphate dehydrogenase (104-base pair protected fragment) and FGF-4 (312-base pair protected fragment) and subsequently subjected to denaturing polyacrylamide gel electrophoresis (6% polyacrylamide, 7 m urea) followed by autoradiography.

Received 5/29/92; accepted 2/22/93.

1 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

2 Supported by NIH Grants R01-CA50376 (F. G. K.) and U01-CA51900 (R. B. D. and M. E. L.). S. W. M. was supported by NIH postdoctoral fellowship CA09218.

3 The abbreviations used are: FGF, fibroblast growth factor; β-gal, β-galactosidase; cDNA, complementary DNA; IMEM, improved minimal essential medium; FBS, fetal bovine serum; CCS, charcoal-stripped calf serum.
**TUMORIGENICITY OF fgf-4-TRANSFECTED MCF-7 CELLS**

**Soft Agar Assay.** The ability of FGF-4-transfected cells to cross-feed SW-13 adrenal carcinoma cells or parental MCF-7 cells in soft agar via secreted FGF-4 was assayed as previously described (5). In brief, feeder cells were plated in 35-mm dishes and allowed to harden. One ml 0.6% bottom agar in IMEM plus 5% serum (FBS or CCS) was plated over the feeder cell layer and allowed to harden. Top agar (0.8 ml, 0.36%) containing 10,000 SW-13 or ML-20 cells in IMEM plus 5% serum (FBS or CCS) was plated over the bottom agar and allowed to harden. For assays in which feeder cell layers were not used, bottom agar was plated in empty dishes, and 10,000 cells were plated in the top layer of agar. Cultures were incubated at 37°C, 5% CO2 for approximately 10 days. Colonies greater than 60 μm were counted using an Omnicron 3600 image analysis system.

**Injection of Nude Mice.** Cells were grown in their normal growth medium to 80–90% confluence and scraped into medium under sterile conditions. Viable cells were counted in a hemocytometer using trypan blue exclusion. Cells were gently centrifuged and cell pellets resuspended in medium so that the desired inoculum was suspended in an injection volume of 0.1 ml. For an inoculum of 10 million cells, the injection volume was 0.15 ml. Breast carcinoma cell lines at doses indicated were injected into the mammary fat pad (4 injections/mouse, 2 on each side) of 6- to 8-week-old ovariectomized athymic nude mice (BALB/c, nu/nu; Charles River), with 4 or 5 mice in each group. Five-mg, 60-day release tamoxifen pellets or 0.72-mg, 60-day release 17β-estradiol pellets (Innovative Research of America, Toledo, OH) were implanted s.c. between the scapulae at the time of injection. In the experiments in which MKS-1 and β-galactosidase producing MCF-7 (MKS-20) cells were coinjected, ovariectomized nude mice were given injections as above of a mixture of 5 million cells of each cell line (10 million cells total) or 5 million cells of one line alone. Measurements were made twice weekly beginning 2 weeks postinjection. Tumor volume was calculated as the product of the largest diameter, the orthogonal measurement, and the tumor depth. Mean tumor volume was calculated as the sum of tumor volumes divided by the number of tumors plus or minus the SEM. Injections that did not produce tumors were not included in the tumor count, but when a tumor regressed, its volume was given as zero in the calculation of the mean.

**Growth Analysis.** Cells maintained in their normal growth medium were changed to phenol red-free IMEM supplemented with 5% CCS and maintained in this medium for 4 days with 4 medium changes during that time. Cells were then plated in the same medium in 24-well plates at a density of 10,000 cells/well and allowed to attach overnight. The next day, day 0, medium was aspirated and treatments were added as indicated. Cells were harvested on appropriate days and counted with a Coulter automated cell counter.

**Staining for β-Gal Activity.** Tumors and organs were fixed in 2% (v/v) formaldehyde/0.2% (v/v) glutaraldehyde in a solution of 137 mM NaCl, 2.7 mM KCl, 10 mM Na2PO4, 1.8 mM KH2PO4, pH 7.4 (PBS) for 2–4 h. The tumors and organs were incubated in a solution of 1 mg/ml 5-bromo-4-chloro-3-indoyl-β-galactoside, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl2, 0.02% Nonidet P-40, and 0.01% sodium deoxycholate in PBS at 4°C overnight. Photomicrographs of whole organs were taken with an Olympus SZH-10 microscope. Tumors and organs were then dehydrated in increasing sucrose concentrations before freezing as described (13). Cryosections were counterstained with eosin, and photomicrographs were taken with an Olympus AH-2 microscope.

**RESULTS**

**FGF-4-transfected MCF-7 Cells Produce Biologically Active FGF-4.** The eukaryotic expression vector pCNCKSEB1 (5) (Fig. 1A), containing the cDNA for fgf-4 under the control of the cytomegalovirus immediate early gene promoter and a second transcriptional unit conferring G418 resistance under control of the same promoter, was transfected into MCF-7 cells. Three clonal cell lines (MKS-1, MKS-13, MKS-15) that express mRNA for FGF-4 were characterized (Fig. 2A). A control clonal cell line (C-1) of MCF-7 cells transfected with the pCNCEB8 expression vector (Fig. 1B) lacking the fgf-4 sequences was also established. To detect biologically active FGF-4 secretion by MKS cells, a feeder layer of MKS cells was plated on the bottom of dishes subsequently plated with soft agar containing a suspension of SW-13 adrenal carcinoma cells. This cell line responds to exogenous FGFs by forming colonies in soft agar (5). SW-13 cells in soft agar without a feeder cell layer have a background level of colony formation that is decreased by the feeder layer of C-1 cells (Fig. 2B). This is a consistent effect of a number of cell lines used as cross-feeding cells, including MCF-7 (data not shown) and, as indicated below, is not limited to SW-13 cells used as indicator cells. Thus, this effect may be due to an inhibitory factor secreted by the feeder cell layer. However, all three of the MKS clonal cell lines, when plated as feeder cells, were able to support significant anchor-age-independent growth of SW-13 cells when compared with the control transfectants (Fig. 2B), demonstrating biological activity of the FGF-4 secreted by the MKS cells.

**FGF-4-transfected MCF-7 Cells Form Tumors in Ovariectomized and Tamoxifen-treated Nude Mice.** The three clonal MKS cell lines were tested for their ability to form tumors when injected into the mammary fat pads of ovariectomized nude mice. Progressively growing tumors were produced by the injection of all three clonal MKS cell lines (Table 1). Six weeks postinjection, with an inoculum of 10 million cells, MKS-1, MKS-13, and MKS-15 cells produced tumors with a mean tumor volume of 404, 23, and 143 mm3, respectively. Control cells at the same time point and with the same inoculum produced barely palpable (mean tumor volume = 5 mm3) static tumors. The MKS-13 cell line, which makes the least amount of fgf-4 mRNA and supports the fewest SW-13 colonies in soft agar, had the lowest tumor incidence and smallest tumor size in vivo, suggesting that the tumorigenicity of each MKS cell line is related to its level of FGF-4 production.

Treatment of nude mice given injections of wild-type MCF-7 cells with s.c. tamoxifen pellets causes a transient stimulation of tumor growth that is followed by a prolonged stationary phase or regression (14). Estrogen treatment under the same circumstances produces progressively growing tumors. Our results with the C-1 control clone or the parental MCF-7 cell lines are in agreement with these published results (Fig. 3A). In contrast, when mice were treated with 5 mg tamoxifen pellets, MKS-1 cells formed tumors that grew more rapidly and to a larger volume than those in untreated ovariectomized mice. Similarly injected mice treated with estradiol pellets developed smaller tumors than untreated mice (Fig. 3B). Comparable results were obtained with mice given injections of MKS-13 cells and treated with estradiol or tamoxifen pellets (data not shown).

**Effect of Estrogen and Tamoxifen on the in Vitro Growth of FGF-4-transfected MCF-7 Cells.** The tumorigenicity of all three FGF-4-transfected cell lines in ovariectomized nude mice and the inhibition of tumor growth by estrogen and tamoxifen stimulation by tamoxifen prompted us to examine their in vitro growth characteristics in response to hormone treatment. In an anchorage-dependent growth assay, MKS-1 cells are growth stimulated by FBS- or estrogen-containing (10 mM 17β-estradiol) medium and growth inhibited under estrogen-depleted conditions (Fig. 4A). This behavior is similar to that of control transfectants or wild-type MCF-7 cells (Fig. 4B). Similar results are obtained with MKS-13 and MKS-15 cells (data not shown). When anchorage-independent growth conditions are used, under estrogen-depleted conditions control transfectants or wild-type MCF-7 cells have a low but detectable rate of colony formation that is increased by estradiol and decreased by 4-hydroxytamoxifen (100 nM) treatment of cultures (Fig. 4C). MKS-1 cells have a higher rate of anchorage-independent growth than parental MCF-7 cells or control transfectants under all conditions. However, estrogen treatment of MKS-1 soft agar cultures stimulates colony formation while tamoxifen treatment inhibits it (Fig. 4C). Similar results were obtained with MKS-13 and MKS-15 cells (data not shown). Thus, in growth assays in vitro, MKS cells exhibit responses to estrogen and tamoxifen that are similar to wild-type MCF-7 cells or control transfectants. How-

Downloaded from cancerres.aacrjournals.org on April 13, 2017. © 1993 American Association for Cancer Research.
ever, MKS cells have increased colony formation in anchorage-independent growth assays when compared with parental cells or control transfectants under all conditions tested. Thus, hormonal growth responses of tumors produced by MKS cell injections are reversed when compared with their in vitro growth and with tumors produced by injection of parental cells or control transfectants.

FGF-4-transfected MCF-7 Cells Are Metastatic. Metastases of s.c. inoculated human breast carcinoma cells in nude mice are typically observed at rather low frequency (15–18). When highly metastatic MCF-7 cells have been observed, they seem to be rare clonal variants (19). Since FGF-4 expression in mouse mammary tumors has been correlated with metastasis, we performed autopsies on 95 tumor-bearing mice given injections of inocula containing MKS cells and 19 tumor-bearing mice given injections of inocula containing only C-1 or native MCF-7 cells. Metastases were detected using hematoxylin and eosin staining of representative sections of axillary and inguinal lymph nodes, spleen, kidney, liver, heart, lung, femur, and brain. Distant metastases were detected in 32 of 136 mice given injections of MKS cells (23%). Metastases were observed in 13 of 56 untreated mice (23%), 7 of 32 estradiol-treated mice (22%), 11 of 41 tamoxifen-treated mice (27%), and one of 7 mice receiving both estrogen and tamoxifen (14%). Of the 19 tumor-bearing mice given injections of either control (C-1) transfectants or wild-type MCF-7 cells, only one metastasis (5.3%) was detected. Metastases were detected in 0 of one untreated mouse, 0 of 5 estrogen-treated mice, one of 12 tamoxifen-treated mice (8.3%), and 0 of one mouse treated with both estrogen and tamoxifen. The lungs were the only metastatic site observed in animals given injections of control cells. Twenty-four of the mice given injections of MKS cells had pulmonary micrometastases, 7 had both pulmonary micrometastases and lymphatic metastases, and 8 had only lymphatic metastases (Fig. 5, A and B). Distant lymphatic metastases were all in the lymphatic tissues of the peritoneal cavity. Three metastases to local axillary or inguinal lymph nodes were also observed. Metastases could be detected at the earliest time point

Fig. 1. Eukaryotic expression vectors used in this study. In A, pCNCKSEBl contains two transcriptional units, each driven by the cytomegalovirus (CMV) immediate early promotor. The first transcriptional unit contains the coding sequence for the bacterial neomycin phosphotransferase gene conferring G418 resistance followed by the herpes simplex virus tyrosine kinase (HSV TK) polyadenylation signal. The fgf-4 cDNA sequences are in the second transcriptional unit and are followed by the simian virus 40 (SV40) polyadenylation signal. The Epstein-Barr virus (EBV) ori P sequences contained in this vector allow the plasmid to be maintained episomally in eukaryotic cells cotransfected with an expression vector for the EBV protein EBNA I. This feature was not used in this transfection. In B, pCNCEB8 is identical to pCNCKSEBl, except that it lacks the cDNA sequences for fgf-4.
**TUMORIGENICITY OF fgf-4-TRANSFECTED MCF-7 CELLS**

Cells were injected s.c. in the doses indicated at 4 sites/mouse. Measurements are from 6 weeks postinjection. Tumor volume is the product of the largest diameter, the orthogonal dimension, and the tumor depth. Mean tumor volume is the sum of tumor volumes divided by the number of tumors ± SEM. Mean tumor volume was significantly different from a similar dose of C-1 cells (Student’s t test).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of cells/injection (millions)</th>
<th>Tumors/10 injections</th>
<th>% take</th>
<th>Mean tumor volume (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>0.1</td>
<td>0/20</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0/20</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>0/20</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0/20</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>6/16</td>
<td>38</td>
<td>5.0 ± 1.6</td>
</tr>
<tr>
<td>MKS-1</td>
<td>0.1</td>
<td>3/20</td>
<td>15</td>
<td>10.7 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>5/20</td>
<td>25</td>
<td>65.4 ± 54.3</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>16/20</td>
<td>80</td>
<td>154.8 ± 71.4</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>16/20</td>
<td>80</td>
<td>249.2 ± 61.3</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>19/20</td>
<td>95</td>
<td>404.0 ± 97.2</td>
</tr>
<tr>
<td>MKS-13</td>
<td>5.0</td>
<td>16/20</td>
<td>80</td>
<td>22.3 ± 5.2</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>8/20</td>
<td>40</td>
<td>23.2 ± 8.4</td>
</tr>
<tr>
<td>MKS-15</td>
<td>5.0</td>
<td>16/20</td>
<td>80</td>
<td>143.2 ± 34.5</td>
</tr>
</tbody>
</table>

* P < 0.10.
* P < 0.05.
* P < 0.005.
* P < 0.001.

FGF-4-transfected MCF-7 Cells Support the Growth of Wild-Type MCF-7 Cells in Ovariectomized and Tamoxifen-treated Nude Mice. Since FGF-4 is a secreted factor, we investigated the possibility that MKS cells would support the growth of an estrogen-dependent cell type in ovariectomized mice or tamoxifen-treated mice. To enable us to distinguish between two different populations of cells within a tumor by histochemical staining, we used native MCF-7 cells expressing the bacterial lacZ gene. A clonal cell line of MCF-7 cells transfected with a plasmid expression vector (pCHCβ-gal) containing coding sequences for the lacZ gene (ML-20 cells) has been described (9). These cells do not differ from native MCF-7 cells with respect to their tumorigenicity in nude mice (data not shown). Mixtures of 5 million MKS-1 and 5 million ML-20 cells were injected s.c. into ovariectomized nude mice with or without estrogen or tamoxifen treatment. MKS-1 or ML-20 cells were also injected alone into ovariectomized nude mice at a dose of 5 million cells/injection. Animals given injections of ML-20 cells received no treatment, estrogen treatment, or tamoxifen treatment.

At 3 weeks postinjection, representative animals were sacrificed. Staining for β-gal activity was performed on the primary tumors and on whole organs removed from the mice. ML-20 cells injected alone formed either small, regressing tumors (tamoxifen treatment) or no tumors at all (no treatment group) but were able to form progressively growing tumors in estrogen-treated mice. The small tumors produced tested, 6 weeks postinoculation. Since each mouse received four injections of tumor cells, which resulted in up to four tumors of different sizes, the appearance of metastases could not be related to primary tumor size. We have subsequently carried out experiments in which each mouse received only one injection of tumor cells. The incidence of metastases in those experiments was correlated with primary tumor size (9).

**Table I Tumors produced by injection of MKS and C-1 cells into ovariectomized nude mice**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of cells/injection (millions)</th>
<th>Tumors/10 injections</th>
<th>% take</th>
<th>Mean tumor volume (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>0.1</td>
<td>0/20</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0/20</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>0/20</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0/20</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>6/16</td>
<td>38</td>
<td>5.0 ± 1.6</td>
</tr>
<tr>
<td>MKS-1</td>
<td>0.1</td>
<td>3/20</td>
<td>15</td>
<td>10.7 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>5/20</td>
<td>25</td>
<td>65.4 ± 54.3</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>16/20</td>
<td>80</td>
<td>154.8 ± 71.4</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>16/20</td>
<td>80</td>
<td>249.2 ± 61.3</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>19/20</td>
<td>95</td>
<td>404.0 ± 97.2</td>
</tr>
<tr>
<td>MKS-13</td>
<td>5.0</td>
<td>16/20</td>
<td>80</td>
<td>22.3 ± 5.2</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>8/20</td>
<td>40</td>
<td>23.2 ± 8.4</td>
</tr>
<tr>
<td>MKS-15</td>
<td>5.0</td>
<td>16/20</td>
<td>80</td>
<td>143.2 ± 34.5</td>
</tr>
</tbody>
</table>

* P < 0.10.
* P < 0.05.
* P < 0.005.
* P < 0.001.
produced by ß-gal expression was not completely homogeneous even in tumors composed only of ß-gal-expressing cells. Therefore, it is difficult to draw exact conclusions concerning the proportion of ß-gal-expressing cells in a particular high-power field of a particular tumor. However, the tumors produced from MKS-1 and ML-20 cell mixtures in estrogen-treated animals contain more blue-staining cells than those from untreated animals, which in turn contain more blue cells than tumors from tamoxifen-treated animals (Fig. 6, C–E). We estimate that even in the tumor from the tamoxifen-treated group, approximately 25% of the tumor cells stain blue. Given the volume of the tumor at the time of sampling (392 mm³), this is too large a fraction to be accounted for solely by the initial injection of ML-20 cells. We conclude that the ML-20 cells in tumors formed by mixed injections in tamoxifen-treated animals are growing and contributing to the volume of the tumor.

ß-Gal expression was also detected in metastases of tumors produced from combination MKS-1–ML-20 injections detected in lymph nodes, brain, lung, and kidney (Fig. 5C), indicating that the ML-20 cells were present in metastases in untreated and tamoxifen-treated animals, as well as estradiol-treated animals. In plastic-embedded sections prepared from the lungs of similarly injected animals in a subsequent experiment, individual metastatic foci were identified which contained either both blue-staining cells and non-blue-staining cells or no blue-staining cells. No foci were identified which contained only blue-stained cells (data not shown). Keeping in mind the caveat concerning consistency of staining, above, and the impossibility of examining every metastatic focus, these data are in agreement with the concept that FGF-4-secreting cells must be in close proximity to the wild-type MCF-7 cells to have a supportive effect. In the first experiment, no metastases were detected in the group of animals injected with only ML-20 cells. Thus, MKS cells are able to support the growth of the ß-gal-expressing MCF-7 cells (ML-20) when they are coinjected into the same site under conditions where MCF-7 cells are not normally capable of growth, i.e., in the absence of estrogen supplementation or in the presence of tamoxifen treatment. In addition, in ovariectomized or tamoxifen-treated ovariectomized mice, MCF-7 (ML-20) cells can be found in metastatic foci from tumors formed when MKS-1 and ML-20 cells are coinjected s.c. (Fig. 5C).

When 5 million MKS-1 cells were injected into the mammary fat pad on one side of ovariectomized nude mice and 5 million wild-type MCF-7 cells were injected into the mammary fat pad on the other side, tumors were observed only on the side injected with the MKS-1 cells. No tumors developed on the side into which the wild-type cells were injected (data not shown). Thus, the effect of FGF-4-secreting cells on ML-20 cells in promoting tumor growth or metastasis in ovariectomized or tamoxifen-treated mice is not due to a systemic effect on the host and presumably involves autocrine or paracrine effects at the tumor site.

MKS Cells Can Cross-Feed Wild-Type MCF-7 Cells in Vitro. In an effort to elucidate the mechanism for the support of wild-type MCF-7 (ML-20) cells when coinjected into the same tumor as the MKS cells, we performed an in vitro experiment designed to reveal cross-feeding of ML-20 cells by MKS cells. Using estrogen-depleted conditions, MKS or ML-20 feeder cells were plated on the bottom of dishes, covered with a layer of bottom agar, and then further covered with a layer of top agar containing ML-20 cells. When supplied with a feeder layer of FGF-4-secreting cells, ML-20 cells increased their rate of anchorage-independent growth significantly (Fig. 7). This effect was not mimicked by a feeder layer of ML-20 cells plated at the same density as the MKS cells (35,000 cells/dish).

To illustrate that the higher rate of colony formation in MKS cross-fed cultures was not due to a faster rate of growth of MKS cells in estrogen-depleted conditions which would produce a higher number of MKS cross-feeding cells at the end of the experiment, the feeder ML-20 cells were also plated at a higher density (50,000 cells/dish).
This plating density produced a confluent feeder layer at the end of the experiment. This increased number of feeder ML-20 cells did not produce an increased number of ML-20 colonies when compared to the lower number of feeder ML-20 cells. When cultures were treated with 100 nM 4-hydroxytamoxifen, MKS cell feeder layers again produced significantly higher colony formation in indicator ML-20 cells than either low- or high-density ML-20 feeder cell layers, although the number of colonies produced by all cross-feeding cells was lower under these conditions when compared with untreated conditions.

Cross-feeding layers of ML-20 cells were actually inhibitory to colony formation when compared with similarly treated cultures which had no cross-feeding cells. MCF-7 cells express the inhibitory transforming growth factor β, and this expression is stimulated by tamoxifen and inhibited by estrogen (20). Thus, under estrogen-depleted or tamoxifen-treated conditions, transforming growth factor β or some other inhibitory growth factor expressed by the ML-20 cells may be inhibiting colony formation below that observed when no feeder cells are used. We feel, therefore, that the important comparison here is between cross-feeding layers of parental cells and transfectants expressing FGF-4.

Thus, it would appear that MKS cells are able to influence MCF-7 (ML-20) cells through a paracrine mechanism in vitro and possibly in vivo when the two cell types are injected into the same tumor. This finding, of course, does not rule out other paracrine mechanisms involving stromal elements, which might be operative in a tumor. Likewise, the results of this experiment do not provide conclusive evidence for a direct paracrine action of FGF-4 on ML-20 cells. It is possible that FGF-4 is modulatory to an inhibitory or stimulatory growth factor which has a direct effect on the indicator ML-20 cells.

DISCUSSION

We have shown that transfection of MCF-7 cells with an expression vector for FGF-4 produces cell lines (MKS cells) which form tumors in ovariectomized nude mice without estrogen supplementation. In addition, these tumors are growth stimulated by tamoxifen treatment of the mice and growth inhibited by estradiol treatment. Thus, hormonal responses of MKS cells in vivo are the opposite of those of the parental MCF-7 cells and the hormonal responses of MKS cells in tissue culture. In addition, tumors produced from MKS cell injections are more frequently metastatic than control transfectants or wild-type MCF-7 cells. MKS cells can support the growth of β-galactosidase-tagged MCF-7 cells when the two cell types are injected into the same tumor and metastases produced from these mixed tumors contain β-gal-expressing MCF-7 cells, indicating that MKS cells are facilitating metastasis of the parental cells.

Production of breast carcinoma cell lines that are tumorigenic in ovariectomized nude mice from previously estrogen-dependent ones has been the goal of many investigators. The rationale behind such attempts has been to mimic the progression of tamoxifen-treated breast tumors from estrogen-dependent to estrogen-independent. Previous transfections of MCF-7 cells with an expression vector for transforming growth factor α (21) have failed to produce cell lines which form progressively growing tumors in ovariectomized nude mice. Transfection of an expression vector for insulin-like growth factor II into MCF-7 cells has been reported to reduce estrogen sensitivity (22) and increase transformed characteristics (23) in vitro. The in vivo tumorigenicity of these transfectants has not been reported.
Transfection of activated ras* into MCF-7 cells produced tumorigenic cell lines in ovariectomized nude mice, but the degree of tumorigenicity did not correlate with ras* expression levels. In addition, estrogen-independent tumors produced from such transfectants are about the same size as tumors produced by control cells in estrogen-treated animals (19, 24, 25) and thus much smaller than tumors produced by MKS cells. Production of breast carcinoma cell lines which do not depend on estrogen for growth from previously estrogen-dependent ones has been accomplished by long-term selection in vitro or in vivo with estrogen-depleted conditions (26–32). In addition, tumors produced by injection of MCF-7 cells into estrogen-treated mice followed by long-term serial transplantation into tamoxifen-treated mice has produced tumor cells which are stimulated by tamoxifen treatment of the mice (33). Very long serial transplantation of these tumors in tamoxifen-treated mice has resulted in tumors which depend upon tamoxifen for growth and which regress if treatment with estradiol is initiated (34). Thus, these tumors share some phenotypic features with the in vivo behavior of MKS cells. Unfortunately, to date, the exact nature of the change(s) responsible for the phenotype of these tumor cells or of cells selected under estrogen-depleted conditions in vitro or in vivo has not been identified. MCF-7 cells selected in estrogen-depleted conditions in vitro (28, 29, 31) as well as the MCF-7 cell derived, tamoxifen-dependent tumors (33, 34) retain estrogen receptors and estrogen responsivity and therefore appear to represent an intermediate in a progression pathway to the full estrogen receptor-negative, antiestrogen-resistant phenotype. MKS cells also retain estrogen and antiestrogen responsivity in vitro (Fig. 4, A and C) and in vivo (Fig. 3B) and may therefore also represent such an intermediate.

Thus, we feel that we have identified one means, transfection of FGF-4, by which MCF-7, an estrogen-dependent cell line, can become tumorigenic in ovariectomized nude mice. The clonal cell lines resulting from this transfection are also more metastatic than the parental ones. However, the mechanism responsible for this change in behavior remains to be elucidated. It has been suggested that the tumorigenic effect produced by FGFs is due to stimulation of neovascularization (6, 35). Increased blood vessel formation might also have a stimulatory effect on the metastatic process (7). If neovascularization is of primary importance in tumor growth or metastasis of MKS tumors, transfection of other angiogenic factors might produce similar results and antiangiogenic compounds might abrogate tumorigenesis in ovariectomized or tamoxifen-treatment mice given injections of MKS cells. Studies are currently under way which address these questions.

Autocrine effects of FGF-4 on the tumor cells themselves might also be responsible for the development of the tumorigenic phenotype of MKS cells in ovariectomized or tamoxifen-treated mice. As mentioned, under some circumstances FGFs are mitogenic for epithelial cells (5, 36) and for MCF-7 cells themselves (37). Thus, FGF-4 may be acting as a mitogen which can replace estrogen stimulation. This view is supported by the ability of cross-feeding MKS cells to stimulate colony formation of ML-20 cells in soft agar and by the ability of MKS cells to support ML-20 cells in tumors injected with MKS-1 and ML-20 cells in ovariectomized and tamoxifen-treated mice. Of course, a modulatory effect of FGF-4 on a stimulatory or inhibitory growth factor could also explain these results.

It is puzzling that tamoxifen has opposite effects on MKS cell growth in vitro and in vivo. One possible explanation for this phenomenon is that in vivo activation of the FGF receptor(s) on MKS cells by secreted FGF-4 may sensitize the cells to the effects of estrogen, in effect shifting the dose-response curve for estrogen to the left. Thus, in tumors, MKS cells would be stimulated by the weak estrogenic activity of tamoxifen and the usual stimulatory dose of estrogen would be inhibitory, as high estrogen concentrations are to MKS-7 cells (29, 38). If this is the case, it is not clear why this effect is not apparent in vitro. In vivo dose-response experiments with estrogen and tamoxifen designed to test this possibility are currently in progress. If this hypothesis were true, we would expect a pure estrogen antagonist to abrogate the stimulatory effects of tamoxifen as well as the inhibitory effects of estradiol in vivo, as is true of tumor cells selected in tamoxifen-treated animals (39). Preliminary experiments with the pure antiestrogen ICI 182,780 are currently under way to test this possibility.

It is not clear whether FGF-4 is directly responsible for the metastatic phenotype of MKS cells or whether metastasis occurs simply because the tumor is larger, more rapidly growing, or more highly vascularized. However, the ability of MKS cells to facilitate the metastatic spread of coinjected wild-type MCF-7 cells transfected with an expression vector for β-gal (ML-20) suggests that secretion of FGF-4 is important in this phenotype. The presence of β-gal-expressing ML-20 cells in metastases in ovariectomized and tamoxifen-treated mice given injections of a mixture of MKS-1 and ML-20 cells could...
suggest that metastasis may be occurring via tumor emboli composed of MKS-1 and ML-20 cells. Alternatively, the FGF-4 secreted from MKS-1 cells in the primary tumor or another metastatic focus could have an effect on a pure ML-20 metastatic focus, enabling the focus to colonize a distant site in an ovariectomized animal. However, the inability of an MKS-1 tumor on one side of the animal to support an MCF-7 tumor on the opposite side argues against FGF-4 secreted from MKS-1 cells having a distant effect. This view is also supported by our inability to identify foci of lung metastasis produced by mixed MKS-1-ML20 injection which contained only blue-staining ML-20 cells. A third possible explanation is that stimulation of FGF receptors on the ML-20 cells by FGF-4 secreted from the MKS-1 cells affects a permanent change in the phenotype of the ML-20 cells while they are still in the tumor, enabling them to colonize distant sites in an ovariectomized animal. It is also possible that the secreted FGF-4 from the MKS-1 cells permits the ML-20 cells to grow in the tumor without estrogen and that they become metastatic simply because of their increased number, just as pure MCF-7 tumors are occasionally metastatic (15–19). Finally, it is also possible that the metastasis of ML-20 cells is facilitated by the neovascularization promoted by the secretion of FGF-4. Studies addressing the mechanism of metastasis are currently under way using β-gal-expressing MKS-1 cells (MKL-4) (9) and ML-20 cells.

FGF expression may be important in the progression of breast tumors from estrogen-dependent to estrogen-independent and in the establishment of the metastatic phenotype. We have observed the presence of mRNA for FGF family members more frequently in estrogen-independent than in estrogen-dependent breast carcinoma cell lines, implying a role for FGFs in breast cancer progression (40). Transcripts or immunoreactivity for various FGFs have been identified in several tumor types (41, 42) and tumor cell lines, including breast (40, 42–45), and may indeed be a mechanism for growth stimulation of these particular tumors. However, in spite of amplification of the chromosomal fragment which contains both the fgf-3 and fgg-4 genes in 15–20% of estrogen receptor-positive breast tumors (44, 46, 47), transcripts for any of the secreted FGFs have been identified only very infrequently in breast tumor specimens (44, 48). However, two FGF family members which lack secretory signal peptides, FGF-1
and FGF-2, are expressed in most tissues (49–51). FGF-2 has been detected in breast (52), and expression of FGF-1 or FGF-2 has been identified in some breast tumors (48). Evidence is accumulating which points to the release of FGF-2 via mechanisms that do not require a secretory signal peptide (53, 54), possibly as the result of tumor progression (35). Many of the FGF receptor variants identified thus far can be activated by multiple members of the family of FGF ligands (55–58). Thus, the results which we have obtained with FGF-4 transfection may mimic effects in breast tumors which are produced by other FGF ligands, possibly FGF-1 or FGF-2. Amplification and overexpression of FGF receptor 1 or FGF receptor 2 has been reported in some breast tumors (48). Evidence is accumulating which points to the release of FGF-2 via mechanisms that do not require a secretory signal peptide (53, 54). Thus, the results which we have obtained with FGF-4 transfection may mimic effects in breast tumors which are produced by other FGF ligands, possibly FGF-1 or FGF-2. Amplification and overexpression of FGF receptor 1 or FGF receptor 2 has been reported in some breast tumors (48).


Fibroblast Growth Factor 4 Transfection of MCF-7 Cells Produces Cell Lines That Are Tumorigenic and Metastatic in Ovariectomized or Tamoxifen-treated Athymic Nude Mice

Sandra W. McLeskey, Junichi Kurebayashi, Susan F. Honig, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/53/9/2168

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.