Quantitative Demonstration of Spontaneous Metastasis by MCF-7 Human Breast Cancer Cells Cotransfected with Fibroblast Growth Factor 4 and LacZ

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ABSTRACT

We recently established transfectedants of MCF-7 human breast cancer cells with fibroblast growth factor 4 (fgf-4) that showed rapid growth and spontaneous metastasis in ovariotomized and tamoxifen-treated nude mice. To establish a spontaneous metastatic model of human breast cancer cells in nude mice with a sensitive marker for detection of micrometastases, the transfection of fgf-4 was combined with transfection of the bacterial lacZ gene encoding ß-galactosidase. MKL-4 cells, a lacZ transfectedant of an fgf-4-transfected cell line, showed the same level of fgf-4 expression as parental cells and expressed a high level of ß-galactosidase activity. When MKL-4 cells were injected s.c. into nude mice, rapidly growing tumors developed. Whole organ staining for ß-galactosidase activity was able to detect even small numbers of metastatic tumor cells. Micrometastases in lymph nodes, lung, and brain were detected 3 weeks after the tumor cell injections, the first time point tested. Within 12 weeks, metastases were observed in lymph nodes, lung, brain, kidney, perirenal fatty tissues, liver, spleen, retroperitoneum, heart, and gallbladder. The frequency of metastasis and number of foci were correlated with the volume of the primary tumors. The distribution of metastatic sites was similar to that in breast cancer patients. MKL-4 cells may be a useful model for studying the malignant progression of hormone-dependent breast cancer, antimetastatic drugs, or early events in metastasis.

INTRODUCTION

Spontaneous metastasis of human breast cancer propagated as solid tumors in nude mice is a relatively rare event (1-3). The reason for this is not known but may relate to the degree of tumor neovascularization in this xenograft system. Neovascularization of human breast cancer has been observed to be correlated with degree of metastatic spread as assessed by lymph node involvement (4). The fibroblast growth factor family has been thought to play an important role in tumor neovascularization and metastasis (5). It has been suggested that expression of the endothelial cell mitogen FGF-4 (6) is associated with the metastatic phenotype in mouse mammary tumors (7) and that amplification of fgf-4 in NIH-3T3 transfectedants is also associated with metastasis (8). Recently, transfection of fgf-4 into the MCF-7 estrogen-dependent human breast cancer cell line resulted not only in rapid tumor growth but also in an increased frequency of detectable spontaneous metastases in ovariotomized and tamoxifen-treated nude mice (9). These transfected breast cancer cells seem to mimic certain aspects of the progression of breast cancer from estrogen dependent to estrogen independent and to be a suitable model for studying events associated with the malignant progression of hormone-dependent breast cancer cells.

Because identification of small numbers of tumor cells in host tissues is difficult, studies on quantification of micrometastases require sensitive markers. Transfection of the bacterial lacZ gene encoding ß-gal into a spontaneously metastatic tumor cell line has been reported to aid in detecting micrometastases in their earliest stage of development (10-13). To establish a spontaneous metastasis model of human breast cancer cells with such a sensitive marker to detect micrometastases, we retransfected the fgf-4-transfected MCF-7 cells, MKS-1 cells (9), with a lacZ expression vector. We established a clonal cell line, MKL-4, which expresses high levels of FGF-4 and ß-gal activity. These cotransfected MKL-4 cells exhibited rapid tumor growth and spontaneous metastasis to axillary and inguinal lymph nodes, lung, brain, kidney, perirenal fatty tissues, liver, spleen, retroperitoneum, heart, and gallbladder in ovariotomized and intact nude mice. Staining for ß-gal activity in excised organs from the host and observation with a dissecting microscope allowed us to detect small numbers of tumor cells and the distribution of the cells in the organs. The MKL-4 cells may be a useful model for studying the malignant progression of hormone-dependent breast cancer cells and the earliest events in metastasis in nude mice. The frequency of metastasis and number of metastatic foci in an organ were positively associated with the volume of the primary tumors. These findings suggest that this metastatic model may provide us with quantitative evaluation of metastasis from the primary tumors. MKL-4 cells may also be useful in the development of antimetastatic drugs.

MATERIALS AND METHODS

Expression Vector Construction. The pCHCß-Gal expression vector (Fig. 1) was constructed using pH1110 (Pharmacia LKB Biotechnology, Piscataway, NJ), which contains the coding sequences for ß-gal, and the pCHC6 expression vector (14), which contains a transcription unit for hygromycin B phosphotransferase, enabling selection of transfectedants in hygromycin B-containing medium. To isolate the lacZ coding sequences for subcloning into the BamHI site of pCHC6, pCH1110 was linearized with HindIII, blunted with Klenow fragment, ligated to BamHI linkers with T4 DNA ligase, and digested with BamHI. PstI was then used to cleave a 3.7-kilobase fragment composed primarily of vector sequences, allowing the isolation of a 3.7-kilobase BamHI fragment that contained the lacZ coding sequences following electrophoresis and electrolution. The pCHC6 expression vector was linearized with BamHI, dephosphorylated with calf intestine alkaline phosphatase, and purified by electrophoresis followed by electrolution. The linearized vector and the lacZ fragment were ligated with T4 DNA ligase and used to transform HB101 competent cells. The final construct was verified to have the correct orientation of the lacZ gene by restriction endonuclease digestion. pCHCß-Gal was purified from bacterial lysate by the lysozyme-alkaline lysis procedure followed by two cycles of CsCl equilibrium density gradient centrifugation as described elsewhere (15).

Transfection. The MKS-1 parental cell line, a transfectedant of FGF-4 into MCF-7 cells which have high levels of FGF-4 expression (9), was transfected with the pCHCß-Gal expression vector or control pCHC6 vector by the calcium phosphate precipitation method of Chen and Okayama (16). Hygromycin B-resistant clonal cell lines were established. lacZ expression was screened by in vitro staining for ß-gal activity described below. Clones expressing high levels of lacZ were assayed for continued fgf-4 mRNA expression by Northern

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analysis and assayed for FGF-4 secretion by a cross-feeding assay described below. MCF-7 cells were also transfected with the pCHCβ-Gal or control vector and screened by the same methods.

Cell Lines. MCF-7 or MKS-1 (9) human breast cancer cells of passage 50-60 were transfected with pCHCβ-Gal or pCHC6 expression vectors. Parental MCF-7 cells and stable transfectants were grown in IMEM supplemented with 5% FCS. Transfectants were selected in IMEM supplemented with 5% FCS and 150 μg/ml of hygromycin B (Boeringer Mannheim Biochemicals, Indianapolis, IN). Two clonal cell lines (MKL-4 and MKL-8) were established from lacZ transfectants of MKS-1 cells. One clonal cell line (ML-20) was established from lacZ transfectants of MCF-7 cells. Two clonal cell lines (C-2 and C-3) were also established as controls from transfectants of MCF-7 and MKS-1 cells, respectively, with pCHC6 expression vector lacking the lacZ fragment. The C-1 clonal cell line, which is a control transfectant for MKS-1 cells, has been previously described (9). SW-13 adrenal carcinoma cells were maintained in IMEM supplemented with 5% FCS.

Animals and Tumors. Five million MKL-4 or ML-20 cells were injected into the right upper mammary fat pad of 4-6-week-old athymic female nude mice (BALB/c-nu/nu). The injection sites were observed once or twice a week for primary tumor appearance and size of the tumors. 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 (±SEM). Injections that did not produce tumors were not included in the tumor count. Several nude mice were sacrificed at 3-week intervals after the tumor cell injections. Primary tumors and selected organs were excised from the mice and cut into 2 pieces for whole organ staining for β-gal activity and formalin fixation, respectively. To analyze the metastatic potential of parental MCF-7 cells, 1 x 10^7 or 5 x 10^6 ML-20 cells, a MCF-7 transfectant with pCHCβ-Gal alone, were similarly injected into the mammary fat pads of ovariectomized nude mice. 17β-Estradiol pellets (0.72 mg, 60-day release; Innovative Research of America, Toledo, OH) were implanted s.c. in the interscapular region at the time of injection. Primary tumors and selected organs were excised at designated times after the injections, and staining for β-gal activity was performed.

Staining for β-Galactosidase Activity. Cultured cells in 35-mm dishes or 12-well dishes (Costar, Cambridge, MA) were rinsed with a solution of 137 mM NaCl, 2.7 mM KCl, 10 mM NaPO4, and 1.8 mM KH2PO4, pH 7.4 (PBS) and fixed for 5 min at 4°C with 2% (v/v) formaldehyde-0.2% (v/v) glutaraldehyde in PBS. The cells were then rinsed with PBS three times and then incubated at 37°C overnight in 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM MgCl2 in PBS. After the staining, the cells were observed and microphotographs taken with an inverted Nikon Diaphot-TMD microscope. In whole organ staining, the excised organs from nude mice were immediately fixed for 2 h at room temperature with 2% (v/v) formaldehyde-0.2% (v/v) glutaraldehyde in PBS. The organs were then incubated with the same 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining solution as above but with 0.02% (v/v) Nonidet P-40 and 0.01% sodium deoxycholate at 4°C overnight. Samples were observed and photomicrographs taken with an Olympus SZH dissecting microscope.

Histology. After staining for β-gal activity, the organs were rinsed with PBS and dehydrated with 70% ethanol followed by 95% ethanol. Sections were cut and stained with hematoxylin and eosin. Photomicrographs were obtained with a Leitz Diaplan digital microscope.

Flow Fluorocytometric Analysis of β-Galactosidase Activity. To assess the clonal hygromycin B-resistant cell lines for homogeneity of β-gal expression, flow fluorocytometric analysis for β-gal activity (17) was performed on the MKL-4, MKL-8, ML-20, and C-2 clonal cell lines. Briefly, the cells were harvested, trypanized, and transferred into a tube at a density of 1 x 10^6 cells in 100 μl of growth medium. The cell suspension was incubated at 37°C for 1 min with 100 μl of 2 mM fluorescein di-β-D-galactoside (Molecular Probes, Eugene, OR) in water. The reaction was stopped by adding 1.8 ml of ice-cold medium. After 1 h of incubation on ice, the cells were filtered through a 30-μm nylon mesh, and the cell suspension was loaded onto a Becton Dickinson dual FACStar flow cytometer.

Northern Analysis. Cytosolic RNA from each cell line was extracted as described elsewhere (18). Briefly, the cells were washed with Tris-buffered saline and incubated with a solution containing 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1.5 mM MgCl2, and 0.65% Nonidet P-40. The mixture was centrifuged and the supernatant was mixed well with a solution containing 7 μl urea, 1% SDS, 0.35 mM NaCl, 10 mM EDTA, and 10 mM Tris-HCl (pH 7.5). The mixture was extracted with phenol:chloroform (1:1, v/v), and cytosolic RNA was precipitated with ethanol. Thirty μg of the total RNA were electrophoresed on a formaldehyde gel (1.2% agarose, 6% formaldehyde) and transferred to a Hybond N nylon membrane (Amersham, Arlington Heights, IL). 32P-labeled antisense riboprobes for FGF-4 and glyceraldehyde 3-phosphate dehydrogenase were prepared as previously described (15). The membrane was incubated with a prehybridization solution (50% [v/v] formamide, 50 mM sodium phosphate, 0.8 M NaCl, 10 mM EDTA, 2.5 X Denhardt’s solution (0.05% Ficol, 0.05% polyvinylpyrrolidone, 0.05% bovine serum albumin), 0.34 mg/ml yeast tRNA, 0.2% SDS, and 0.4 mg/ml sonicated salmon sperm DNA) at 65°C for 2 h. Then the membrane was incubated with a hybridization solution with the same constituents plus approximately 1 x 10^6 dpm of each riboprobe/ml at 65°C overnight. After hybridization, the membrane was washed 3 times with a solution containing 0.1% SDS, 150 mM NaCl, and 15 mM sodium citrate, pH 7.0 (0.1% SDS/0.1 X standard saline citrate) (150 mM NaCl, 15 mM sodium citrate) at 75°C for 30 min for each wash. The membrane was autoradiographed at ~80°C overnight.

Cross-Feeding Assay for Quantification of FGF-4 Secretion. Ten thousand cells of each cell line were plated as feeder cells in 35-mm dishes and allowed to grow overnight. The secretion of FGF-4 was quantified with the FGF-sensitive SW-13 adrenal carcinoma cell line as previously described (9, 15). One ml bottom agar and 0.8 ml top agar in IMEM plus 5% FCS containing 1 x 10^6 SW-13 cells were plated over the feeder cell layer. Cultures were incubated at 37°C, 5% CO2 for 10 days. Colonies greater than 60 μm were quantified using an Omnicron 3600 image analysis system.

RESULTS

lacZ Transfectants Express High Levels of β-Galactosidase Activity. Hygromycin B-resistant clonal cell lines were established after transfection with the pCHCβ-Gal plasmid (Fig. 1). This plasmid contains the lacZ coding sequence under the control of the cytomegalovirus immediate early promoter. The control cell lines were established using the pCHC6 vector lacking the lacZ sequences. Each cell line was screened for β-gal activity by in vitro β-gal staining. Transfection of MKS-1 cells with pCHCβ-Gal yielded one clonal cell line, MKL-4 (Fig. 2A) and MKL-8, showing strongly positive staining in vitro. Transfection of MCF-7 cells with pCHCβ-Gal yielded one clonal cell line, ML-20, that showed strong positive staining for β-gal activity in vitro (Fig. 2C). Clonal cell lines C-2, a transfectant of

![Diagram of lacZ expression vector]
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Fig. 2. Phase contrast microscopic analysis of in vitro β-gal staining in MKS-1 or MCF-7 transfectants of pCHCβ-Gal or control plasmid. Semiconfluent cultures of each cell line were stained by the in vitro β-gal staining method and observed with a phase contrast microscope (original, × 100). A, MKL-4 cells (a clonal cell line of MKS-1 cells transfected with pCHCβ-gal). B, C-3 cells (a clonal cell line of MKS-1 cells transfected with control pCHC6). C, ML-20 cells (a clonal cell line of MCF-7 cells transfected with pCHCβ-Gal). D, C-2 clonal cell line (MCF-7 transfected with control pCHC6).

control pCHC6 into MCF-7 cells (Fig. 2D), and C-3, a transfectant of control pCHC6 into MKS-1 cells (Fig. 2B), were also established by selecting for hygromycin B resistance. As expected, no blue stain was observed with these cells.

As shown in Fig. 3, the β-gal activity of two MKL cell lines and the ML-20 cell line were analyzed by flow fluorocytometric analysis using a fluorogenic substrate for the enzyme. Both of the cell lines showed high levels of β-gal activity when compared with the control C-2 cell line. The MKL-4 cell line showed 30 times higher β-gal activity than the MKL-8 cell line. The two peaks of activity observed with MKL-4 cells were subsequently found to be due to cell clusters (data not shown). Therefore, MKL-4 cells are homogeneous in their staining intensity. The ML-20 cells also showed high, homogeneous levels of β-gal activity.

To be certain that the secondary transfection did not influence the expression of initially transfected FGF-4 vector, we analyzed the two MKL cell lines for FGF-4 expression. Northern analysis of FGF-4 mRNA showed that both MKL-4 and MKL-8 cell lines have the same level of FGF-4 expression as the parental MKS-1 cell line (Fig. 4). A cross-feeding assay in which the FGF-sensitive adrenal carcinoma cell line SW-13 serves as an indicator for secreted FGF activity also showed that both of the secondary transfected cell lines secrete the same level of biologically active FGF-4 as the parental MKS-1 cells (Fig. 5).

Since we hypothesized that higher expression of β-gal in tumor cells may help us detect a smaller number of tumor cells in host tissues, the highest expressor of β-gal activity, the MKL-4 cell line, was selected for use in nude mouse experiments.

MKL-4 Cells Can Be Detected in Primary Tumors and Metastatic Sites. After injection of five million MKL-4 cells into reproductively intact, athymic female nude mice, rapidly growing tumors developed in 80% of the animals (34 of 45) (Fig. 6). Conversely, ML-20 cells showed no tumors (0 of 5) in similar nude mice as expected, since the animals did not receive estrogen supplementation (19).

The MKL-4 tumors in nude mice grew rapidly, and central necrosis was rarely observed. FGF-4 secretion from the tumor cells is thought to promote angiogenesis. Quantitative assay of neovascularization and vascular structures surrounding the tumor tissues is currently under investigation.

Several nude mice were sacrificed every 3 weeks after the injections of MKL-4 cells. Primary tumors, axillary and inguinal lymph nodes, brain, lungs, heart, liver, gallbladder, spleen, and kidneys were routinely removed. The excised organs were stained for β-gal activity as described. The staining rendered tumor cells strongly blue within 15 min. No color change was observed in normal host tissues. After an overnight incubation with the staining solution, lymph nodes, kidneys, and gallbladder became pale green, but blue-stained tumor cells were...
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These observations were supported by findings in frozen sections of primary tumors and metastatic lesions counterstained with eosin (Fig. 8, A–C). The cytoplasm of large tumor cells in the metastatic foci were strongly blue stained. Because of the diffusion of the stain from the tumor cells, some lymphocytes beside the tumor cells were pale blue in the lymph node. However, no strongly blue-stained host cells were observed in the sections. Furthermore, cultures have been established in vitro from minced lymph nodes or lung tissues that were both G418 and hygromycin B resistant (data not shown), confirming the presence of the MKL-4 cells in these tissues.

Fig. 4. Northern analysis of fgf-4 or glyceraldehyde 3-phosphate dehydrogenase mRNA in the parental MKS-1 cell line, and two MKS-1 and one MCF-7 transfectant with pCHCß-Gal. Two parallel samples of 30 μg cytosolic RNA of each cell line were electrophoresed on a denaturing gel and transferred onto a nylon membrane. The membrane was hybridized with 32P-labeled antisense fgf-4 (Lanes 1–4) or glyceraldehyde 3-phosphate dehydrogenase (Lanes 5–8) riboprobe. Lanes 1 and 5, ML-20 cells; Lanes 2 and 6, MKL-4 cells; Lanes 3 and 7, MKL-8 cells; Lanes 4 and 8, MKS-1 parental cells.

Fig. 5. Cross-feeding assay for quantification of FGF-4 secretion in parental MKS-1 cell line, MCF-7, or MKS-1 transfectants of pCHCß-Gal or control plasmid. FGF-sensitive SW-13 adrenal carcinoma cells in soft agar were cross-fed by feeder cells of each cell line. Numbers of colonies greater than 60 μm were quantified with an Omnicon 3600 image analysis system. Values represent the mean number of colonies/dish. Bars, SD.
Multiple lung micrometastases were observed in nude mice with primary tumors more than 100 mm³ in volume. Many tumor clusters were spread throughout the lung tissues (Fig. 7D). Interestingly, various patterns of micrometastases or tumor emboli, such as solitary, grouped, or linear patterns, were observed in the lung (Fig. 7E). These findings suggest that lung micrometastases in this model can be quantified and may be useful for studying early events in lung metastasis. In intact mice, micrometastases in spleen, liver, heart, and gallbladder developed only in nude mice with primary tumors more than 500 mm³ in volume. These results suggest that metastases in those organs may be late events in this model. In a separate experiment, however, untreated ovariectomized mice given injections of 10⁷ ML-20 cells exhibited liver and spleen metastases at 3 weeks postinjection even though the tumors in these animals were much smaller than 500 mm³ (Table 3). This result may reflect experimental variation. However, we have shown that these tumors are inhibited by estrogen (9), so it is possible that metastasis may be favored in ovariectomized animals when compared with the reproductively intact animals used in the first experiment.

Frequency of Metastasis and Number of Metastatic Foci Were Associated with Volume of ML-4 Primary Tumors. Because ML-4 tumors did not grow homogeneously (Fig. 6), the relationships between duration of time after tumor cell injections and frequency of metastasis were not evident (Table 1). Micrometastases in lymph nodes, lung, and brain could be detected as early as 3 weeks after tumor cell injections. In contrast, micrometastases in kidney, spleen, and liver were observed only 9 weeks after the injections. We sacrificed 17 mice to terminate the animal experiments 12 weeks after tumor cell injections. In contrast, micrometastases in kidney, spleen, and liver were observed only 9 weeks after the injections. We sacrificed 17 mice to terminate the animal experiments 12 weeks after tumor cell injections. In the remaining 8 mice (mean primary tumor volume, 100 mm³ in volume but in 8 of 13 (61.5%) nude mice with primary tumors more than 100 mm³ in volume. Micrometastases in ipsilateral lymph nodes had already developed in 7 of 20 (35%) nude mice with primary tumors less than 100 mm³ in volume. Metastasis in ipsilateral axillary lymph nodes is the most frequent event (55%, 18 of 33). Interestingly, micrometastases in contralateral lymph nodes, lung, and brain were observed in the remaining 8 mice (mean primary tumor volume, 100 mm³ in volume but in 8 of 13 (61.5%) nude mice with primary tumors more than 100 mm³ in volume. Micrometastases in brain developed in 4 of 20 (20%) mice with primary tumors less than 100 mm³ in volume and in 6 of 13 (46.2%) mice with primary tumors more than 100 mm³ in volume. Thus, there were positive relationships between frequency of micrometastases in lung and brain and volume of the primary tumors. Furthermore, the number of micrometastatic foci in lung (mean ± SE, 26.6 ± 8.7/organ) and brain (7.6 ± 2.8/organ) of nude mice with primary tumors more than 100 mm³ in volume was significantly larger than that in lung (0.6 ± 0.3/organ) and brain (0.3 ± 0.2/organ) of nude mice with primary tumors less than 100 mm³ in volume (P < 0.05 and P < 0.05, respectively, by Student’s t test).

β-Galactosidase Expression in Wild-Type MCF-7 Cells Also Confers an Increased Ability to Detect Micrometastases at Early Time Points. The frequency of spontaneous metastasis we observed when random, staged pathologic sections from animals injected with FGFR-producing MKS-1 cells were examined microscopically was less than we observed with ML-4 cells that produce both β-galactosidase and FGFR-4 (9). We also detected metastases in MKL-4-inoculated mice at sites where metastases were not found following the examination of stained sections of the same organs from MKS-1-inoculated mice. In that study, ovariectomized mice received a larger inoculum of cells than in the present study, making difficult a direct comparison with the data reported here using intact female mice and a smaller inoculum. Nevertheless, we attribute the increased frequency reported in this study to the fact that the 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside staining facilitates the examination of the whole organ or tissue rather than limiting the examination for metastasis to random sections. These data raised the possibility that β-galactosidase expression in MCF-7 cells that did not overexpress FGFR-4 would also facilitate the detection of metastasis. We therefore injected 10⁷ ML-20 cells s.c. into estradiol-treated ovariectomized nude mice and observed them for metastases. Three weeks after inoculation, four tumor-bearing mice with a mean tumor volume of 28 ± 12 had only one local lymphatic metastasis and eight distant ones (Table 3). The ML-20 metastases consisted of only single or very few cells. In contrast, when 10⁷ ML-4 cells were injected into ovariectomized mice as part of the same experiment, at three weeks metastases were observed at all sites at a higher frequency, and half of the lymphatic metastases were multicellular (Table 3).

In two other experiments estrogen-supplemented ovariectomized mice that had been inoculated with ML-20 cells were examined for metastases at later time points. In one experiment, the two tumor-bearing mice of the five mice that were given injections of 10⁷ ML-20...
cells at each of four sites exhibited no metastases when examined 6 weeks after inoculation. Both mice bore at least one tumor that was greater than 100 mm$^3$. In the second experiment, the two tumor-bearing mice of the four that were inoculated with $5 \times 10^6$ ML-20 cells at four sites also failed to exhibit metastases when examined 57 and 71 days after inoculation. This was despite the fact that the animal that was examined at 57 days bore a tumor that was 126 mm$^3$ and the animal examined at 71 days harbored two tumors that were 306 and 325 mm$^3$ in size. In contrast, in other experiments all MKL-4-injected animals that bore tumors $>100$ mm$^3$ showed metastasis (Table 2).

**DISCUSSION**

Transfection of oncogenes or growth factor-related genes into normal or low-malignant potential tumor cells is an attractive tool for studying tumor cell progression to a more malignant or especially to
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Fig. 8. Microscopic analysis of histological sections of MKL-4 primary tumor and metastases in a lymph node and lung. The samples were excised from nude mice and stained by whole organ staining for β-gal. Then the samples were washed with PBS, dehydrated, and frozen. Cryosections were counterstained with eosin. A, a primary tumor (original, × 100). Note the homogeneously blue-stained tumor cells. B, metastasis in a lymph node (original, X 100). Note the aggregated tumor cells. C, a solitary micrometastasis in a lung (original, × 100).

a metastatic phenotype. Transfections of oncogenes such as activated (mutant) ras, the H-ras-family, serine-threonine kinases (v-mos and v-raf), tyrosine kinases (v-src, v-fes, and v-fms) and mutant p53 have been reported to induce or increase metastatic potential in the appropriate recipient cells (reviewed by Liotta et al., Ref. 20).

Tumor angiogenesis is thought to be a critical factor in the metastatic phenotype (4, 5). FGF-4 has angiogenic activity and is secreted, enabling it to function as a paracrine activator of endothelial cells (6, 21). FGF-4 expression has been reported to be positively associated with metastatic phenotype in mouse mammary tumors and NIH-3T3 cells (7, 8). Recently, we established FGF-4 transfectants of MCF-7 cells and reported that this transfection induces the cells to grow rapidly and progressively in ovariectomized athymic nude mice without estrogen supplementation. Moreover, spontaneous microscopic and/or macroscopic metastases were observed in 23% of mice after an injection of the MKS-1 clonal cell line, one of the transfectants (9).

Spontaneous metastasis of human tumor xenografts in athymic nude mice has been reported mainly for melanoma and colorectal carcinoma cells and sporadically for renal, bladder, gastric, prostatic, pancreatic, endometrial, lung, and breast carcinomas and some sarcoma cells (1–3, 22–37). Development of spontaneous metastasis requires not only late events (formation of tumor emboli, extravasation into the surrounding stroma and parenchyma, establishment of a new growth) but also early events (tumor cell detachment, migration, intravasation). Therefore, a spontaneously metastatic model is likely to be more useful for developing antimetastatic drugs than an experimental metastasis model using i.v. injections of a tumor cell suspension, since this spontaneously metastatic model would also allow the testing of agents targeted at some of the early events in the process. Heretofore, the low frequency and prolonged latency of metastasis of s.c. injected breast cancer cells precluded effective study of events important in spontaneous metastasis. Thus, a breast cancer cell line which has an ability to spontaneously metastasize has been reported to assist in detecting micrometastasis without any other phenotypic changes (10–13).

Our goal in this experiment was to establish a reliable, spontaneously metastatic model of human breast cancer cells with a sensitive marker to detect micrometastasis in nude mice. We also expected that such an experimental model might provide us with a stable screening system for antimetastatic drugs. Therefore, we established a clonal

Table 1 Time course of microscopic and/or macroscopic metastasis in explored organs of nude mice given injections of MKL-4 cells

<table>
<thead>
<tr>
<th>No. of mice</th>
<th>3 weeks</th>
<th>4–6 weeks</th>
<th>7–9 weeks</th>
<th>10–12 weeks</th>
</tr>
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<tbody>
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<td>Tumor volume</td>
<td>55.6 ± 28.8</td>
<td>115.4 ± 42.4</td>
<td>408.8 ± 146.3</td>
<td>474.7 ± 172.9</td>
</tr>
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Five million MKL-4 cells were injected into the right upper mammary fat pad of 4–6-week-old athymic female nude mice. Several nude mice were sacrificed every 3 weeks after the injection. Seventeen mice were sacrificed 12 weeks after the injections to terminate the experiments. Selected organs were excised and stained by the whole organ staining method and observed with a dissecting microscope.

ipsilateral axillary lymph nodes.

d Frequency of macroscopic metastasis.

e Contralateral axillary or inguinal lymph nodes.
cell line, termed MKL-4, a stable transfectant of MCF-7 human breast cancer cells with fgf-4 and lacZ.

The MKL-4 clonal cell line was shown to have the same level of FGF-4 expression as the parental MKS-1 cell line and had the highest level of β-gal expression of the pCHCβ-Gal transfecants. Transfection of the bacterial β-gal gene into MKS-1 cells dramatically assisted us in detecting small numbers of tumor cells in host tissues. Although microscopic and/or macroscopic metastases were recognized in only 33 (66.7%) nude mice injected with MKL-4 cells. Although we could recognize microscopic and/or macroscopic métastases in 22 of 33 (66.7%) nude mice injected with MKL-4 cells. Although we could not microscopically recognize brain, kidney, spleen, and liver of the nude mice was positively associated with volume of the primary tumors (Table 2). Furthermore, the number of micrometastatic foci in brain or lung was significantly correlated with volume of the primary tumors, as described in “Results.”

Because the in vivo growth patterns of the primary tumors differ between MKL-4 cells and ML-20 cells, a clonal line of MCF-7 cells transfected with the pCHCβgal vector, it is difficult to make a direct comparison of the metastatic potential of the two cell lines. Like wild-type MCF-7 cells, ML-20 cells form tumors in ovariectomized athymic nude mice only when supplemented with estrogen, and these tumors are relatively slow growing. In contrast, the FGF-4-overexpressing MKL-4 cells form rapidly growing tumors in the absence of exogenous estrogen, and primary tumors are paradoxically inhibited by exogenous estrogen (9). In most of this study, we used intact female nude mice that did not receive estrogen supplementation. Under these conditions, both tumorigenicity and metastasis were observed only in the FGF-4-overexpressing MKL-4 cells and not in the mice given injections of ML-20 cells. This reliable occurrence of metastases with MKL-4 cells under these conditions points out their potential utility as a metastatic model for the development of antimeetastatic agents.

Metastases were observed in mice inoculated with ML-20 cells that received estrogen supplementation but at a lower frequency than was observed in untreated MKL-4 inoculated mice and only at early time points. These results suggest that ML-20 cells are unlikely to have the same metastatic potential as MKL-4 cells and that the FGF-4 expression in the MKL-4 cells is an important factor in the increased frequency of metastases observed. It remains an open question, however, as to whether FGF-4 expression is a qualitative determinant of the increased frequency of metastasis or merely an enhancing factor that allows metastases to be detected earlier. FGF-4 is an angiogenic factor that may provide a stimulus for metastatic growth.

Table 2: Relationships between primary tumor volume and frequency of microscopic and/or macroscopic metastasis in explored organs of nude mice injected with MKL-4 cells

<table>
<thead>
<tr>
<th>Tumor volume (mm³)</th>
<th>No. of mice</th>
<th>Regional a</th>
<th>Distal a</th>
<th>Lung</th>
<th>Brain</th>
<th>Kidney</th>
<th>Spleen</th>
<th>Liver</th>
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<tr>
<td>&lt;100</td>
<td>20 (9)</td>
<td>7 (1)</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100-1000</td>
<td>9 (9)</td>
<td>7 (4)</td>
<td>2 (2)</td>
<td>5</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>&gt;1000</td>
<td>4 (6)</td>
<td>4 (3)</td>
<td>4 (4)</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

* Five million MKL-4 cells were injected into the right upper mammary fat pad of 4-6-week-old athymic female nude mice. Several nude mice were sacrificed every 3 weeks after the injection. Selected organs were excised and stained by the whole organ staining method and observed with a dissecting microscope.

The MKL-4 clonal cell line was shown to have the same level of FGF-4 expression as the parental MKS-1 cell line and had the highest level of β-gal expression of the pCHCβ-Gal transfecants. Transfection of the bacterial β-gal gene into MKS-1 cells dramatically assisted us in detecting small numbers of tumor cells in host tissues. Although microscopic and/or macroscopic metastases were recognized in only 33 (66.7%) nude mice injected with MKL-4 cells. Although we could recognize microscopic and/or macroscopic métastases in only 22 of 33 (66.7%) nude mice injected with MKL-4 cells. Although we could not microscopically recognize brain, kidney, spleen, and liver of the nude mice was positively associated with volume of the primary tumors (Table 2). Furthermore, the number of micrometastatic foci in brain or lung was significantly correlated with volume of the primary tumors, as described in “Results.”

Because the in vivo growth patterns of the primary tumors differ between MKL-4 cells and ML-20 cells, a clonal line of MCF-7 cells transfected with the pCHCβgal vector, it is difficult to make a direct comparison of the metastatic potential of the two cell lines. Like wild-type MCF-7 cells, ML-20 cells form tumors in ovariectomized athymic nude mice only when supplemented with estrogen, and these tumors are relatively slow growing. In contrast, the FGF-4-overexpressing MKL-4 cells form rapidly growing tumors in the absence of exogenous estrogen, and primary tumors are paradoxically inhibited by exogenous estrogen (9). In most of this study, we used intact female nude mice that did not receive estrogen supplementation. Under these conditions, both tumorigenicity and metastasis were observed only in the FGF-4-overexpressing MKL-4 cells and not in the mice given injections of ML-20 cells. This reliable occurrence of metastases with MKL-4 cells under these conditions points out their potential utility as a metastatic model for the development of antimeetastatic agents.

Metastases were observed in mice inoculated with ML-20 cells that received estrogen supplementation but at a lower frequency than was observed in untreated MKL-4 inoculated mice and only at early time points. These results suggest that ML-20 cells are unlikely to have the same metastatic potential as MKL-4 cells and that the FGF-4 expression in the MKL-4 cells is an important factor in the increased frequency of metastases observed. It remains an open question, however, as to whether FGF-4 expression is a qualitative determinant of the increased frequency of metastasis or merely an enhancing factor that allows metastases to be detected earlier. FGF-4 is an angiogenic factor that may provide a stimulus for metastatic growth.

Table 3: Frequency and extent of metastases in nude mice given injection of β-galactosidase-expressing breast cancer cell lines

Ovariectomized mice were given injections of 10 million cells in one site. Tumors and organs were harvested 3 weeks later.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor volume (mm³)</th>
<th>Ipsilateral lymph node</th>
<th>Dist lymph node</th>
<th>Lung</th>
<th>Brain</th>
<th>Kidney</th>
<th>Spleen</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>MKL-4 (no treatment)</td>
<td>4</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>B</td>
<td>123</td>
<td>++</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>20</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>D</td>
<td>54</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>E</td>
<td>225</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MTV a = 85 ± 40 (SEM)</td>
<td>TOTAL 3</td>
<td>3</td>
<td>3</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>ML-20 (E2 treatment)</td>
<td>12.6</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>B</td>
<td>29.4</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>62.4</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>D</td>
<td>8.4</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>MTV b = 28 ± 12 (SEM)</td>
<td>TOTAL 1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

* MTV = sum of all tumor volumes/no. of tumors. +, few diffuse blue spots (fewer than about 15) or one focus of a few blue spots; ++, diffuse blue spots (about 5-15) or several foci of a few blue spots.

** E2 treatment consisted of a 0.72 mg, 60-day release 17β-oestradiol pellet implanted s.c.
growth factor, and increased angiogenesis is related to increased metastasis in human breast cancer (4). On the other hand, we have observed that the number of metastases and their frequency of occurrence at distant sites appears to be related to the size of the primary tumor. Therefore, the increased frequency of detection of metastasis with the MKL-4 cells compared to that observed with the ML-20 cells may be related to the faster growth rate of the MKL-4 tumors. It is possible that with extended periods of tumor growth, metastases may also be observed with a higher frequency in ML-20-inoculated mice. This, however, does not appear to be the case. Four estrogen-supplemented mice bearing ML-20 tumors did not have metastasis when examined 6–10 weeks after injection despite bearing tumors of a size that was a reliable indicator of the presence of metastases in MKL-4-inoculated mice. This absence of metastases at later time points suggests that ML-20 cells, in the small numbers observed in lymph nodes and organs at 3 weeks postinjection, lack the ability to grow and are eventually cleared. This may also be the case for the small MKL-4 tumors which produced microscopic liver and spleen metastases at a 3-week time point (Table 3) when at a later time point, only MKL-4 tumors greater than 500 mm³ produced such metastases. In any case, with MKL-4-injected animals, the correlation of the appearance of macroscopic foci with increased primary tumor volume suggests that at least some MKL-4 cells have the potential to continue to grow at metastatic sites. We have not yet analyzed sufficient numbers of estrogen-supplemented mice inoculated with MKL-4 cells that bear tumors of a sufficient size to allow us to determine if there is a hormonal component to a possible disappearance of metastases at later time points as observed with ML-20 cells. As stated above, this analysis is complicated by the inhibitory effect of exogenous estrogen on MKL-4 primary tumor growth. Nevertheless, it is precisely the reliably high frequency of metastases at early time points and their sustained presence in ovariectomized mice not receiving exogenous estrogen that is the useful feature of the MKL-4 metastatic model.

The distribution of metastatic sites in this model is surprisingly similar to that in breast cancer patients. Ipsilateral axillary lymph nodes are the most common metastatic sites in human breast cancer. Metastases in ipsilateral axillary lymph nodes were also the most frequent events in this model. Other organs, such as lungs, brain, liver, and kidneys, are relatively common metastatic sites of human breast cancers in our model (38). The similarity of distribution of metastatic sites suggests that this spontaneous metastatic model of human breast cancer may be a useful tool for studying malignant progression of breast cancer, especially with relation to metastasis. The few other breast cancer cell lines which can metastasize spontaneously in nude mice have been reported to develop metastasis with long latency in lymph nodes, lung, brain, pleura, peritoneum, liver, spleen, heart, and spine (1–3). However, no detailed findings of micrometastasis and metastatic cell distribution in organs have been reported.

MKL-4 cells have been transfected with drug-resistance genes for hygromycin B and G418. These drug-resistance genes are useful for eliminating the growth of host cells in culture. To establish MKL-4 subclones derived from metastatic lesions, in vitro culture of metastatic lymph nodes and lungs using hygromycin B and G418 containing-media is under investigation. We are currently attempting to determine whether these subclones either have a stronger metastatic potential than the parental MKL-4 cells or exhibit preferential metastasis to specific sites.

In conclusion, cotransfection of the fgf-4 and lacZ genes into the MCF-7 human breast cancer cell line has provided a reliable, spontaneous metastatic model with an extremely sensitive marker for detecting single tumor cells in host organs of athymic nude mice. The frequency of metastasis and number of metastatic foci were positively related to primary tumor volume, especially in the lymph nodes, lungs, and brain of the nude mice. The distribution of metastatic sites in this model was surprisingly similar to that in human breast cancers in women. These results suggest that this spontaneously metastatic model of human breast cancer may be useful not only for preclinical study of antimetastatic drugs but also for fundamental study of the earliest stages of metastasis in human breast cancer.

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