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Five Novel Hormone-responsive Cell Lines Derived from Murine Mammary Ductal Carcinomas: *In Vivo* and *in Vitro* Effects of Estrogens and Progestins[1]

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ABSTRACT

We have developed an experimental model of mammary carcinogenesis in which the administration of medroxyprogesterone acetate (MPA) to female BALB/c mice induces progestin-dependent ductal metastatic mammary tumors with high levels of estrogen receptor (ER) and progesterone receptor (PR). Through selective transplants in untreated mice, we have obtained progestin-independent variants, still expressing high levels of ER and PR. Primary cultures of the MPA-induced carcinomas C4-HD and C7-HI were set up, and after 3–4 months, several different cell lines were obtained. Four of these, MC4-L1, MC4-L2, MC4-L3, and MC4-L5 were established from C4-HD and a fifth, MC7-L1, from C7-HI. All cells were of epithelial origin, as demonstrated by electron microscopy and by immunocytochemical identification of cytokeratin and cadherin. *In vitro* MC4-L1, MC4-L3, and MC4-L5 showed a typical epithelial morphology; when transplanted *in vivo*, they originated metastatic carcinomas with different degrees of differentiation. MC4-L2 and MC7-L1 deviated from the standard epithelial picture; they disclosed a spindle-shaped morphology *in vitro* and *in vivo* gave rise to a biphasic spindle cell/tubular carcinoma and an anaplastic carcinoma, respectively; both lines gave rise to metastases. This differential morphology correlated with a higher degree of aggressiveness, as compared with MC4-L1, MC4-L3, and MC4-L5. ERs and PRs were detected by binding, immunocytochemistry, and Western blot. *In vitro*, MC4-L2 and MC7-L1 were stimulated by MPA (nM to μM) and 17β-estradiol (nM and 10 nM); no significant stimulation was observed in MC4-L1, MC4-L3, and MC4-L5 under the same experimental conditions. *In vivo*, MPA significantly stimulated tumor growth in all epithelioid lines but not in MC4-L2 and MC7-L1. A progestin-dependent growth pattern was confirmed for MC4-L1, MC4-L3, and MC4-L5 in successive transplants, whereas MC4-L2 and MC7-L1 behaved as progestin independent. This is the first description of mouse mammary carcinoma cell lines expressing ER and PR. The different *in vitro* hormone responses as compared with *in vivo* and the differential effects of 17β-estradiol in the parental tumors and in cell lines render these lines useful tools for the *in vitro* and *in vivo* study of hormone regulation of tumor growth and metastases.

INTRODUCTION

Breast cancer is the most common form of cancer among women in North and South America, Europe, and Australia, accounting for approximately 15–18% of the deaths in these populations (1–3). Its incidence is increasing by ~1% per year in both industrialized and developing countries (4), and it is estimated that the disease will affect ~5 million women within the next decade (5). The etiology of breast cancer remains largely unknown, and despite the development of different therapeutic approaches, the mortality rate has been continuously rising over the past 30 years. With the increase in the age of the female population, prevention and treatment of breast cancer will continue to represent a major challenge.

The dilemma of mammary tumor development and the mechanisms related to tumor growth have been studied using different experimental approaches. Mouse mammary tumors induced by the mouse mammary tumor virus represent a suboptimal model of human disease. Although viral sequences have been reported recently in human breast tumors, thus far no virus has been demonstrated to be involved in the genesis of human mammary carcinomas (6). The virus-induced tumors in mice either do not respond to hormones or express steroid hormone receptors and are absolutely pregnancy dependent (7, 8). The chemical carcinogen models allowed the dissection of initiators and promoters. The tumors originated in both the *N*-methyl-*N*-nitrosourea and the 7,12-dimethylbenz[*a*]anthracene rat models are hormone responsive, and they express ERs[3] (9, 10). They do not, however, give rise to metastases. They harbor point mutations in oncogenes that are not mutated in human disease. These mutations are in general specific for the chemical carcinogen used (11).

Another approach to the study of this problem is the use of established cell lines. The most widely used human breast cancer cell lines are MCF-7 (12), T-47D (13), and ZR-75-1 (14). They all express ERs and PRs and are hormone responsive. Other cell lines with similar features have been established recently (15–19). Key features of a human cancer model, such as the ability to give rise to metastases when inoculated in immunosuppressed mice, are, however, absent from most xenografted cell lines, unless specifically manipulated (20).

Few mouse models have been used to study the role of hormone regulation in tumor growth. The MXT model is a mammary tumor that originated in F_1(C57 × DBAf), which is maintained by syngeneic transplantation and expresses high levels of ER and PR (21). We have developed an experimental model in which ductal progestin-dependent metastatic mammary carcinomas are induced by the continuous administration of MPA to BALB/c female mice (22). These tumors express high levels of ER and PR (23) and are maintained through serial syngeneic passages in MPA-treated mice. By transplantation into untreated mice, we have been able to generate progestin-independent tumor lines that retain the expression of ER and PR (24). Using primary and secondary cultures derived from C4-HD, one of the progestin-dependent tumor lines, we were able to demonstrate that MPA stimulates cell proliferation directly and that E_2 and antiprogestins inhibit cell growth, even at very low concentrations (25, 26).

With the aim of further dissecting some aspects of the hormonal response, we had previously developed a technique to obtain purified epithelial or fibroblastic primary cultures from these tumors (25). Although primary cultures are an excellent tool to study direct effects of hormones on cell proliferation, the approach is time consuming because to bypass the inherent heterogeneity of the primary culture and to standardize our findings, many different controls need to be run

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[3] The abbreviations used are: ER, estrogen receptor; PR, progesterone receptor; MPA, medroxyprogesterone acetate; E_2, 17β-estradiol; ssFCS, steroid-stripped FCS; WM, washing medium; SM, standard medium.

in parallel. To overcome these shortcomings, we developed cell lines to study several parameters associated with hormone responsiveness. We obtained four cell lines derived from one progestin-dependent tumor and one from a progestin-independent tumor. This represents the first description of mouse mammary adenocarcinoma cell lines obtained in nontransgenic animals expressing ER and PR. The hormone receptor expression, the *in vivo* and *in vitro* hormone responses, and the lack of need for immunosuppressed animals to evaluate *in vivo* effects render our model a useful tool to evaluate mechanisms related to the effect of hormones and antihormones on cell proliferation.

MATERIALS AND METHODS

Hormones

MPA and E_2 were obtained from Sigma Chemical Co. (St. Louis MO) and were dissolved in absolute ethanol at 10^{-3} M (stock solution). Working solutions were freshly prepared before each experiment. In *in vivo* experiments, MPA depot (Medrosterona; Laboratorio Gador, Buenos Aires, Argentina) was used.

ssFCS

To strip the sera of steroids, activated charcoal (Mallinckrodt Chemical Works, New York, NY) was added to FCS (Life Technologies, Inc., Gaithersburg, MD or Gen Sociedad Anónima, Buenos Aires, Argentina) to a final concentration of 0.05 g/ml. The extraction was carried out at 4°C overnight. Charcoal was removed by five consecutive centrifugations at 10,000 rpm for 15 min. The procedure was repeated twice, the second time for 3 h, to increase the efficiency of the stripping.

Culture Media

DMEM/F-12 [1:1, without phenol red (Sigma Chemical Co.)], 100 units/ml penicillin, and 100 μg/ml streptomycin. WM was DMEM/F-12 + 5% FCS. SM was DMEM/F-12 + 5% ssFCS.

Primary Cultures

Tumors were aseptically removed, minced, washed with DMEM/F-12, suspended in 5 ml of enzymatic solution [2.5 mg/ml trypsin (Life Technologies, Inc.); 5 mg/ml albumin (Life Technologies, Inc.); and 850 units/ml collagenase type II (Life Technologies, Inc.) in PBS] and incubated at 37°C for 20 min under continuous stirring. The liquid phase of the suspension was then removed, and the undigested tissue was incubated for an additional 20 min with fresh enzymatic solution. Enzyme action was interrupted by adding WM. Epithelial and fibroblastic cells were separated by a modification of the sedimentation technique as described previously (25). Briefly, the cells were resuspended in another 20 ml of WM and allowed to precipitate for 20 min. The upper 15 ml were discarded, the cells in the sediment were resuspended in other 20 ml of WM and allowed to precipitate for 20 min, and the procedure was repeated for ~10 times. The cells were plated in culture flasks with SM and allowed to attach for 24–48 h. The medium was then removed and replaced by fresh medium with 10^{-8} M MPA. The medium was changed every 2–3 days. At confluence or when cell clusters looked overcrowded, the cells were detached with 0.25% trypsin, washed, and resuspended in fresh SM.

Establishment of Cell Lines and Culture Conditions

Two ductal MPA-induced carcinomas were used to obtain the cell lines; C4-HD, a progestin-dependent tumor (27) at passage 60, which has been maintained by syngeneic transplantation in MPA-treated mice; and C7-HI, a progestin-independent tumor (27) at passage 50, which has also been maintained by syngeneic transplantation but in untreated female mice. The main characteristics of both lines are shown in Table 1.

The epithelial enriched cultures growing in the presence of SM and 10 nM MPA were incubated in DMEM/F-12 with the addition of 10% FCS (Life Technologies, Inc.), 2 mM glutamine (Life Technologies, Inc.), 2 μg/ml bovine insulin (Life Technologies, Inc.), 100 units/ml of penicillin/100 μg/ml streptomycin, amphotericin B (Life Technologies, Inc.), 10 nM tiroxin (Sigma Chemical Co.), 0.3 μM cortisol (Sigma Chemical Co.), 10 ng/ml transferrin (Sigma Chemical Co.), and 10 nM MPA. About 20 days after seeding, a proportion of epithelial cells became vacuolated and started to detach; fibroblasts, which were very scanty at the beginning, increased in number. During the next 2–3 months, one of the most conspicuous features was the appearance of giant multinucleated epithelial-like cells, a feature that was almost predictive of cell line establishment.

During the time of cell line establishment, the cultures had to be successively concentrated because of cell loss. When the passages were performed, the cells were subcultured into smaller wells. The fibroblasts were successively purged from the culture; the cells that were unable to attach during the first or second hours were removed and transferred to another well, and the procedure was repeated twice. Most cell lines arose from epithelial clusters present in the fast-attaching cell populations.

During the first 3–4 months, about five or six subcultures were performed, and from each subculture fewer and fewer cells were harvested. The first morphological change suggestive of the establishment of a line was the appearance of colonies composed of small uniform cells. Four cell lines originated from three different C4-HD primary cultures. MC4-L2 is a subline derived from passage seven of MC4-L1 in which a small group of cells remained attached after trypsinization; with time this subculture expanded, growing with a morphological pattern completely different from that of the parental cells. A similar phenomenon occurred with MC7-L1. The cell line arose from wells where the general morphological aspect was of scanty epithelial cells intermingled with a mass of fibroblast-like cells.

Doubling Time

The doubling time of the cell lines *in vitro* was determined by plating the cells in six-well plates at a concentration of 20,000 cells/well and counting duplicate wells at 9 a.m. and 5 p.m. for 1 week. The values were calculated from the log phase of the growth curves.

Table 1 *Parental tumor line features: C4-HD and C7-HI*

Histology	C4-HD[a]	C7-HI[a]
Hormone dependence	Progestin dependent: grows in progestin-treated mice. Inhibited by E_2.	Progestin independent: grows in untreated mice. Inhibited by E_2.
Metastases	Lung, 4–5 months after tumor transplantation	Lungs and axillary homolateral lymph nodes, 1–2 months after tumor transplantation
Receptor binding (fmol/mg prot)		
Estrogen (mean ± SD)	Cytosol: 65 ± 20 (n = 6)	Cytosol: 34 ± 8 (n = 6)
Progesterone mean (range)	Cytosol: 253 (133–637) (n = 6)	Cytosol: 131 (15–393) (n = 7)
	Nuclear: 453 (404–503) (n = 2)	Nuclear: 615: (356–845) (n = 4)
Glucocorticoid	Negative	Negative
Androgen	Negative	Negative
Epidermal growth factor	Negative	Negative
c-erbB2 amplification	Yes	No
Cell lines originated	MC4-L1, MC4-L2, MC4-L3, MC4-L5	MC7-L1

[a] Infiltrating ductal carcinomas that originated in 1985 in BALB/c mice treated with 40 mg of MPA depot every 3 months (23), Group C, cage 4 for C4-HD and cage 7 for C7-HI. The HD or HI ending was added in accordance with the ability to grow in hormone-treated or untreated animals, respectively (hormone-dependent or hormone-independent).

Immunohistochemistry

Cell lines were grown on eight-well chamber slides or Leighton tubes. For routine H&E staining and immunocytochemistry, the slides were rinsed three times in PBS and fixed for 45 min in 10% buffered formalin (for morphology, c-erbB2, and hormone receptors) or in ethanol (for cytokeratins and vimentin). Tumor tissue was fixed in 10% buffered formalin (for ERs and PRs) or ethanol (for cytokeratins) and embedded in paraffin using standard methods. Four-μm sections were obtained and stained with H&E for histopathology. All immunostainings were performed with the ABC method using the Vectastain Elite ABC immunoperoxidase system (Vector Laboratories, Burlingame, CA), as described by the manufacturer. For cytokeratins, a polyclonal rabbit antibody was used (Z0622; Dako Corp., Carpinteria, CA) at 1:250 dilution. ER (MC-20) and PR (SC-20) rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). MC-20 was diluted 1:50 for tissue sections studies and 1:100 for chamber slides, and SC20 was diluted 1:100 for tissue sections and 1:200 for chamber slides. PR monoclonal antibody (1:250 in chamber slides) was purchased from Neomarkers (Freemont, CA). Staining was developed with 0.060% 3,3′-diaminobenzidine (Sigma Co.). For immunostaining of cytoplasmic antigens such as the cytokeratins, the cells were occasionally counterstained with hematoxylin. Negative controls were performed by replacing the primary antibody with normal rabbit serum.

Electron Microscopy

Cell monolayers were fixed with 4% paraformaldehyde and 1% glutaraldehyde in cacodylate buffer, postfixed in osmium tetroxide, and routinely embedded in Vestopal. Sections were cut with glass knives, stained with uranyl acetate and lead citrate, and observed in a Zeiss EM-109-T electron microscope at 80 kV.

Cytogenetics

Semiconfluent cultures were treated with 0.1 μg/ml Colcemid (Life Technologies, Inc.) for 2 h at 37°C and detached with trypsin. Hypotonic treatment was performed in 0.075 M potassium chloride for 10 min at 37°C, and the cells were fixed with 3:1 methanol:glacial acetic acid. The slides were stained with 3% Giemsa (Sigma Chemical Co.). The following passages were used: MC4-L1, 22; MC4-L2, 19; MC4-L3, 28; MC4-L5, 20; and MC7-L1, 26. The chromosome number is expressed as the modal number, the number of chromosomes most frequently found after analyzing at least 100 metaphases.

ERs and PRs

The presence of ER and PR was evaluated by immunocytochemistry as described above, by ligand binding using the whole cell technique at single saturation points and by Western blot using cell extracts.

Whole Cell Assay. Cells (10^5) were plated in 24-well plates with complete medium. After 3 days, whole-cell PR and ER assays were performed as described previously (25). Briefly, a total of 300,000 cpm of 17α-methyl-[^{3}H]R5020 (DuPont NEN, Boston, MA; specific activity, 85 Ci/mmol) were added together with a 100-fold excess of R5020 or ethanol for PR or 300,000 cpm of [^{3}H]E_2 (DuPont NEN; specific activity, 86 Ci/mmol) together with 100-fold excess of E_2 or ethanol for ER. After 2 h of incubation, the cells were washed, trypsinized, and counted in a liquid scintillation counter. A significant difference between the experimental groups, those incubated only with radioactive hormone and those incubated with radioactive plus unlabeled hormone, yields the total cpm bound to the receptors.

Preparation of Cytosolic Extracts. Cell lines were harvested with a rubber policeman and placed in buffer A [20 mM Tris-HCl (pH 7.4), 1.5 mM EDTA, 0.25 mM DTT, 20 mM Na_2MoO_4, and 10% glycerol]. Protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.025 mM *N*-CBZ-L-phenylanine chloromethyl ketone, 0.0025 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, 0.025 mM *N*-tosyl-L-phenylalanine chloromethyl ketone, and 0.025 mM *N*-α-*p*-tosyl-L-arginine methyl ester) were added before preparing the extracts. The homogenate was sonicated twice at medium frequency for 10 s in ice and centrifuged for 20 min at 12,000 rpm at 4°C. The supernatant was immediately stored at −70°C or in liquid nitrogen and used later in the immunoblot assays. Protein concentration was determined by Lowry *et al.* (28).

Western Blot Analysis. Equal amounts of proteins (100 μg/lane) were separated on discontinuous 7.5% (for PR) or 12% (for ER) polyacrylamide gels (29). A set of prestained molecular weight standards was run on each gel. Proteins were dissolved in sample buffer [6 mM Tris (pH 6.8), 2% SDS, 0.002% bromphenol blue, 20% glycerol, and 5% mercaptoethanol] and boiled for 4 min. After electrophoresis, proteins were blotted to a nitrocellulose membrane. The membranes were blocked overnight with 5% dry skimmed milk dissolved in 0.1% PBST [0.8% NaCl, 0.02% KCl, 0.144% Na_2PO_4, 0.024% KH_2PO_4 (pH 7.4), and 0.1% Tween 20], washed several times with PBST, and probed with PR Ab-7 (2 μg/ml) or ER MC-20 (1 μg/ml) in PBST at room temperature for 2 h. The blots were washed three times, 10 min each, and probed with peroxidase-conjugated sheep antimouse immunoglobulin (for PR) or peroxidase-conjugated donkey-antirabbit immunoglobulin (for ER; Amersham Life Science, Buckinghamshire, United Kingdom). The luminescent signal was visualized with the ECL Western blotting detection reagent kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), and exposed to a CUPRIX RP 1 (Medical X-ray film; Agfa) for 1–5 min. Western blots were performed from the MC4-L1 line, passages 24, 92, and 95; MC4-L2, passages 16, 45, and 62; MC4-L3, passages 20 and 26; MC4-L5, passages 9 and 20; and MC7-L1, passages 18 and 41. Uteri obtained from mice primed with 10 μg/kg E_2 and NMuMG cells (gently provided by J. C. Calvo, Instituto de Biología y Medicina Experimental) were used as positive and negative controls, respectively. NMuMG cells are epithelial cells derived from mouse normal mammary glands (30).

Tumorigenicity

Cells were trypsinized and resuspended in 10-fold excess culture medium (with 10% FCS). After centrifugation, cells were resuspended in serum-free medium, and 10^6 cells were injected s.c. in a final volume of 0.1 ml using a 21-gauge needle in the right inguinal flank of ovariectomized and nonovariectomized BALB/c mice that had been inoculated contralaterally with 40 mg of MPA depot (n = 6/group). The mice were examined every 3 days. When tumor size was >400 mm^2, the animals were sacrificed, and a complete autopsy was performed. These tumors were maintained by syngeneic passages in two MPA-treated and two untreated female BALB/c mice. After asserting MPA responsiveness, tumors growing in MPA-treated mice were chosen for the next passage. All *in vivo* experiments were carried out using BALB/c mice, and animal care was in agreement with institutional guidelines.

Effect of MPA and E_2 on Cell Proliferation: [^{3}H]Thymidine Uptake Assay

In a Corning 96-well microplate, 0.1 ml/well of a cell suspension were seeded in SM at a concentration of 10^5 cell/ml. After attachment (24 h), the cells were incubated for 72 h with the experimental solutions to be tested (MPA, 0.1 nM to 1 μM; E_2, 0.1 nM to 1 μM; in 1% ssFCS). Fifty % of the medium was replaced with fresh medium every 24 h. The cells were incubated with 0.4 μCi of [^{3}H]thymidine (specific activity, 20 Ci/mmol) for 24 h, trypsinized, and harvested in a cell harvester. Filters were counted in a liquid scintillation counter. The assays were performed in octuplicates, and mean and SD were calculated for each solution tested.

Statistical Analysis

The differences between controls and experimental groups in [^{3}H]thymidine uptake assays were analyzed by ANOVA, followed by the Tukey t test between groups. Differences in receptor levels between spindle and epithelioid cells were determined using the Student t test.

RESULTS

Morphology

In Vitro

The five cell lines grew as monolayers. MC4-L1, MC4-L3, and MC4-L5 disclosed a typical epithelial morphology, although in overgrown cultures, groups with more than one layer were observed. MC4-L1 cultures showed a more heterogeneous picture, with giant multinucleated cells, that were observed even after 100 passages (Fig.

1*A*). MC4-L3 and MC4-L5 were morphologically more homogeneous. MC4-L2 and MC7-L1 cultures were composed of spindle-shaped, fibroblastic-like cells (Fig. 1, *E* and *I*). Both lines disclosed lower adhesion to the substrate as compared with the other epithelial-like lines, because they could be very easily detached with trypsin-EDTA.

In Vivo

MC4-L1. When injected *in vivo*, this cell line grew as an infiltrating ductal carcinoma resembling the parental tumor, showing a cribiform and tubular growth pattern, with areas of solid growth (Fig. 2*A*). Glandular differentiation in the cribiform areas often showed signs of secretion. The proliferating cells were polygonal, with ovoid, pale nuclei containing one or more eosinophilic nucleoli. Numerous mitotic and apoptotic images were observed. In large tumors, necrosis as well as inflammatory infiltration was always present. Occasionally, necrosis was located in the center of neoplastic structures, resembling a comedocarcinoma. The stroma was scanty and well vascularized. The tumor metastasized to the lung (Fig. 2*B*). In metastases, the histology was somewhat less differentiated than in the primary tumor.

MC4-L2. These cells, when injected into syngeneic mice, developed tumors disclosing a biphasic growth pattern, with sarcomatoid areas showing no morphological signs of epithelial differentiation, composed of very atypical spindle-shaped cells, forming irregular groups that infiltrated and dissociated the surrounding tissue, mimicking a fibroblastic sarcoma (Fig. 2*F*, *right*); the nuclei were bizarre with irregularly distributed chromatin, and one or more nucleoli and groups of mononuclear cells resembling mast cells could be observed frequently. The cells were frequently multinucleated (Fig. 2*F*, *white arrow*). Other areas showed a definite carcinomatous growth pattern

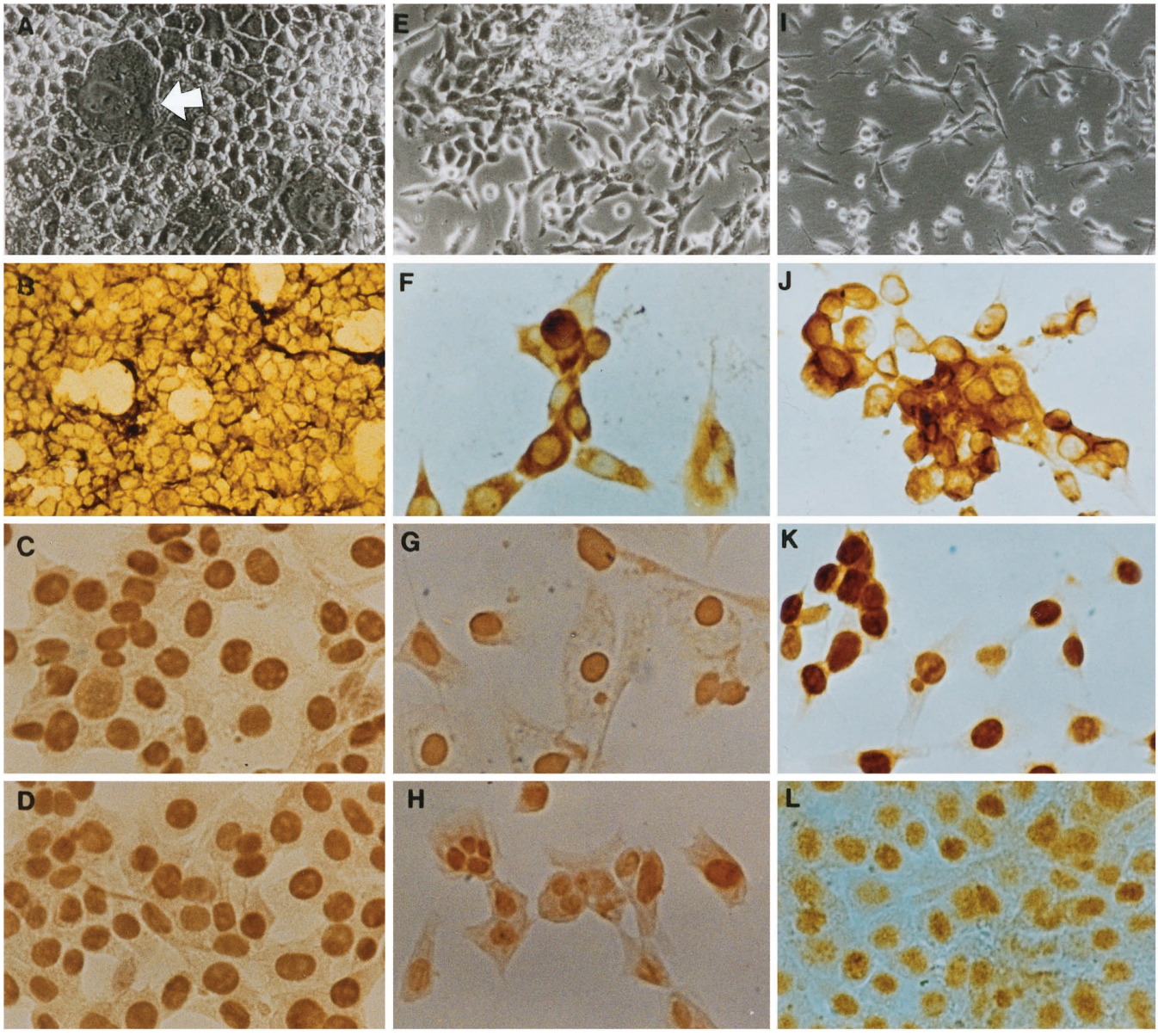

Fig. 1. Cell lines growing *in vitro*. Morphological and immunohistochemical studies were performed on cells from passages 15 to 20. All steroid receptor immunohistochemical studies were performed using MC-20 and SC-20 antibodies. MC4-L1: *A*, confluent culture disclosing a typical epithelial morphology. Giant cells are evident (*arrow*; phase contrast, ×200); *B*, all cells disclose positive cytoplasmic immunoreactivity for cytokeratins (×200); *C* and *D*, nuclear immunoreactivity for ER and PR, respectively (×400). MC4-L2: *E*, the cells disclose a fibroblast-like appearance in culture (phase contrast, ×200); *F*, all cells show positive cytoplasmic immunoreactivity for cytokeratins (×400). *G* and *H*, nuclear immunoreactivity for ER and PR, respectively (×400). MC7-L1: *I*, intermingled round and fusiform cells are characteristic of this MC7-L1 culture (phase contrast, ×100); *J*, all cells are positive for cytokeratins (×400). *K* and *L*, nuclear immunoreactivity for ERs and PRs, respectively (×400).

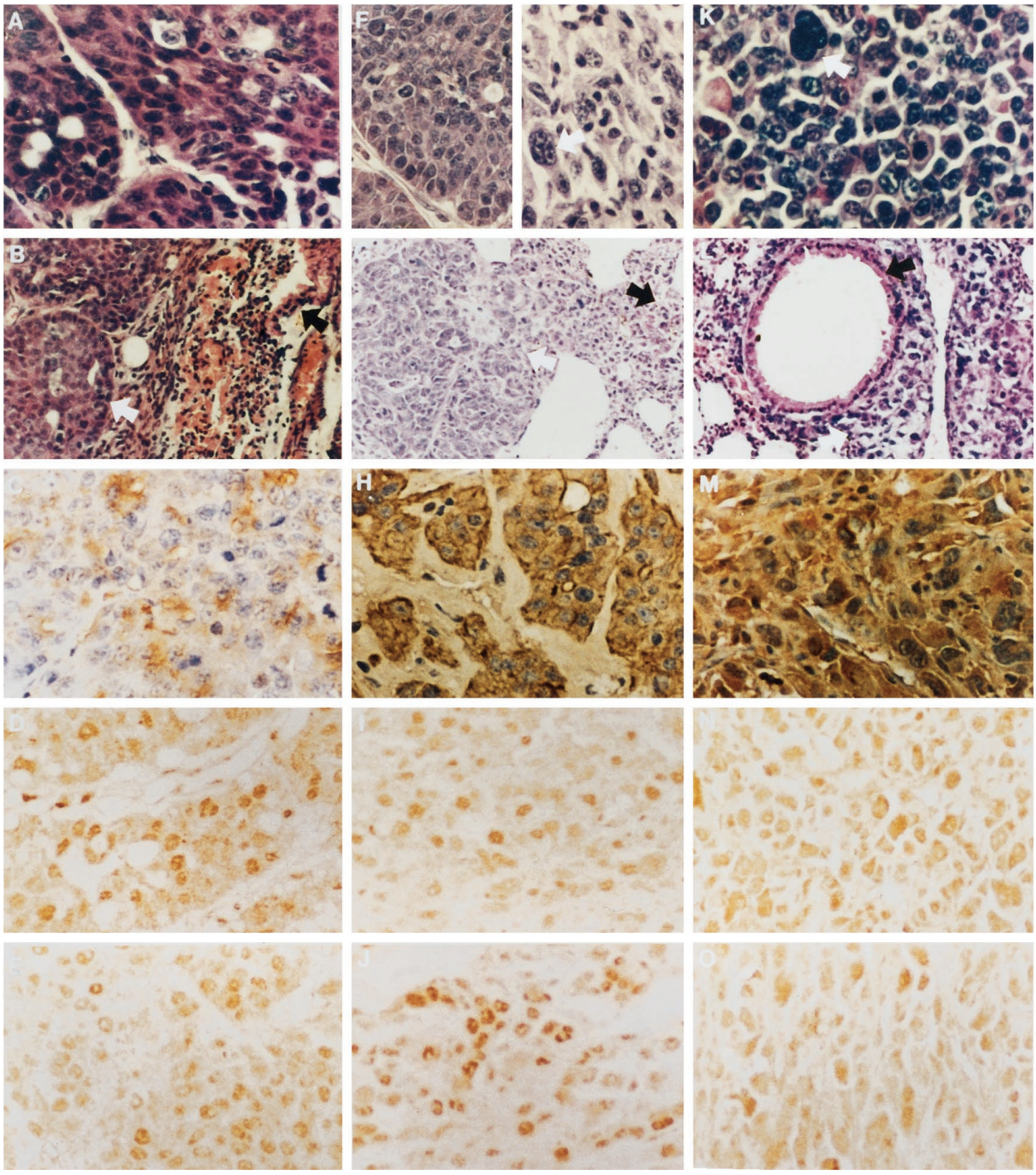

Fig. 2. Histology of the cell lines and their metastases growing *in vivo* in BALB/c female mice treated with MPA and immunohistochemical patterns of expression of different proteins. MC4-L1: *A*, high-power view of a moderately differentiated adenocarcinoma with occasional glandular differentiation. The stroma is scanty and well vascularized (H&E, ×400); *B*, metastatic MC4-L1 adenocarcinoma. The tumor (*left area*, *white arrow*) infiltrates the lung parenchyma. *Black arrow*, remaining bronchial epithelium (H&E, ×200). *C*, moderate-to-strong cytoplasmic immunoreactivity to cytokeratins is observed in epithelial cells (hematoxylin counterstain, ×400). ER (*D*) and PR (*E*) immunoreactivity is located in the nuclei of the malignant cells (×400). MC4-L2: *F*, this carcinoma discloses a biphasic pattern with fusiform (*right*) and epithelioid (*left*) areas. *Arrow*, a multinucleated giant cell, a frequent finding in fusiform areas (H&E, ×400). *G*, lung metastasis. The proliferating malignant cells (*white arrow*) infiltrate and compress the lung parenchyma (*black arrow*; H&E, ×200). *H*, all malignant cells are positive for cytokeratin (hematoxylin counterstain, ×400). More than 80% of the epithelial malignant cells are immunoreactive for ER (*I*) and PR (*J*; ×400). MC7-L1: *K*, no glandular differentiation is evident in this tumor, disclosing the histological picture of an anaplastic carcinoma, with frequent multinucleated giant cells (*white arrow*; H&E, ×100); *L*, the metastatic tumor (*white arrow*) grows along a vascular wall, surrounding a bronchial structure (*black arrow*; H&E, ×40). The malignant cells are positive for cytokeratin, confirming their epithelial origin (*M*; hematoxylin counterstain, ×400). Nuclear immunoreactivity for ER (*N*) and PR (*O*) in a high percentage of the epithelial malignant cells (×400) is shown.

with solid groups and cords of polygonal cells, occasionally disclosing glandular differentiation (Fig. 2*F, left*). The stroma was scanty, although well vascularized. The lesion infiltrated extensively the surrounding tissues and gave rise to lymph node and lung metastases (Fig. 2*G*).

MC4-L3. This cell line grew *in vivo* as an infiltrating solid ductal carcinoma, with few or absent signs of glandular differentiation. The proliferating cells were big and polygonal, with ovoid and pale nuclei with one or no nucleoli and clear cytoplasm. The stroma was abundant and fibroblastic. In large tumors, necrosis and thrombosis were almost

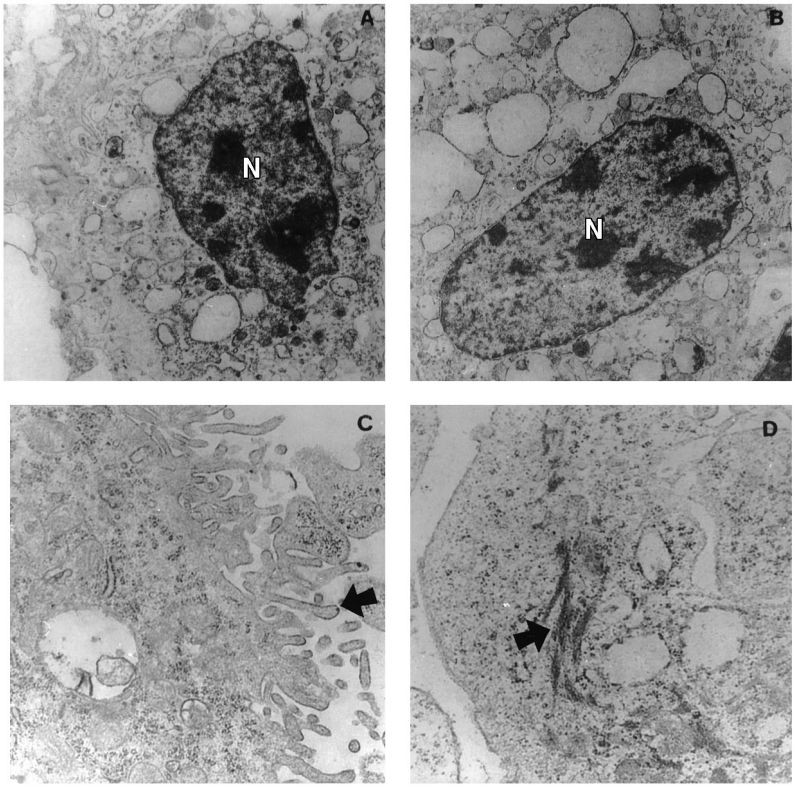

Fig. 3. Electron microscopy of MC4-L2. *A* and *B*, well-developed rough endoplasmic reticulum as well as smooth vesicles are observed (*N*, nucleus). *C*, polarized cell with abundant microvilli (*arrow*) and free ribosomes. *D*, the presence of tonofilaments (*arrow*) confirms the epithelial nature of the cell line. ×17,000.

always present. In some areas, especially in the periphery of the tumor, cell pleomorphism was more evident. Numerous mitotic and apoptotic images were observed.

MC4-L5. This cell line, when inoculated *in vivo*, grew as an infiltrating ductal carcinoma showing solid groups of proliferating cells with occasional glandular differentiation. The neoplastic cells were polygonal, disclosing big ovoid nuclei with irregularly distributed chromatin, one nucleolus, and scanty cytoplasm. Extensive areas of necrosis were observed in almost all large tumors. Numerous mitotic figures were observed as well as cells with signs of apoptosis. The stroma was scanty. The tumor grew rapidly and metastasized to regional lymph nodes.

MC7-L1. The tumor grew as a very aggressive anaplastic carcinoma with no signs of glandular differentiation (Fig. 2*K*). It grew rapidly, extensively infiltrating the s.c. tissue, muscle, and peritoneum, reaching the kidneys, where it showed a somewhat lymphoma-like pattern of infiltration. The proliferating cells were very atypical, with giant multinucleated cells with dark irregular nuclei. The mitotic index was very high. The tumor metastasized to lung (Fig. 2*L*) and regional lymph nodes. The morphology in lung metastases showed areas more differentiated than the primary tumor.

Cell Differentiation

The five cell lines revealed cytoplasmic staining for cytokeratins, both *in vitro* (Fig. 1, *B, F,* and *J*) and *in vivo* (Fig. 2, *C, H,* and *M*), demonstrating their epithelial nature despite their morphological differences. The degree of reactivity was more heterogeneous in *in vivo* growing tumors than in cells growing in culture, which was possibly attributable to technical reasons. Vimentin was evaluated in MC4-L3 and in MC7-L1 and was slightly positive in both lines, a frequent finding in epithelial cells growing *in vitro*. All lines expressed cadherin with different degrees of intensity (not shown).

Electron Microscopy

All of the cell lines showed a similar ultrastructural pattern, confirming their epithelial origin. Large intracytoplasmic vacuoles and tonofilaments were observed consistently, whereas microvilli were detected in MC4-L1, MC4-L3, and MC4-L2. This last finding suggests that at least some cells are polarized *in vitro*. Retroviral particles were never detected. Representative images of these electron microscopic features are shown in Fig. 3.

Hormone Receptors

Cells

ERs and PRs were studied by immunocytochemistry, binding techniques at saturating points, and by Western blot in cell cultures. Both receptors were detected in all cell lines.

Immunocytochemical specific nuclear staining for both ER and PR is shown in Fig. 1, *C* and *D, G* and *H,* and *K* and *L*. By binding techniques, ER and PR levels (Table 2) were higher in the spindle-shaped lines (MC4-L2 and MC7-L1, pooled data, mean ± SE; ER, 14.6 ± 2.3; PR, 130 ± 20.1, fmol/mg protein) than in polygonal cell lines (MC4-L1, MC4-L3, and MC4-L5, pooled data, mean ± SE; ER, 4.39 ± 1.5; PR, 13.78 ± 6.42, fmol/mg protein; ER, $P < 0.05$; PR, $P < 0.001$). PR isoforms A (M_r 83,000) and B (M_r 115,000), and ERα (M_r 66,000) were expressed in all cell lines in both early and late passages. PR is detected with a higher intensity in MC4-L2 and in MC7-L1 as compared with MC4-L1. No specific ER and PR bands were seen in NMuMG cells. Representative Western blots are shown in Fig. 4.

Tumors

All tumors growing *in vivo* showed positive nuclear staining for both ER and PR (Fig. 2, *D* and *E, I* and *J,* and *N* and *O*), although with

Table 2 *Main features of the cell lines*

	MC4-L1	MC4-L2	MC4-L3	MC4-L5	MC7-L1
Parental tumor	C4-HD	C4-HD	C4-HD	C4-HD	C7-HI
In vitro morphology	Polygonal	Spindle shaped	Polygonal	Polygonal	Spindle shaped
Detachment	>10 min.	<1 min	>10 min	>10 min	<1 min
Duplication time (h)	24.48	18.68	25.45	25.02	20
Modal chromosome number (range)	68 (60–75)	68 (59–74)	61 (54–70)	36 (33–39)	74 (64–85)
ER (fmol/10^5 cells)[a]	1.27–5.6	20.8–9.6	2.69	8	13.8–14.2
ER (IMH)[b]	+++[c]	+++	+++	+++	+++
PR (fmol/10^5 cells)[a]	3.65–13.48	145–164	6	32	141–72
PR (IMH)	+++	+++	+++	+++	+++
Cytokeratin *in vitro*	Positive	Positive	Positive	Positive	Positive
c-erbB2 *in vitro* (IMH)	++++	++++	++++	ND[d]	+
Cadherin E	Positive	Positive	Positive	Positive	Positive
Tumor morphology	Ductal carcinoma	Biphasic carcinoma	Ductal carcinoma	Ductal carcinoma	Anaplastic carcinoma
Local invasion	Poor	High	Poor	Poor	Very high
Metastases	Lung	Axillary lymph nodes and lung	Lung	Axillary lymph nodes and lung	Axillary lymph nodes and lung
ER and PR in tumor sections (IMH)	+++	+++	+++	+++	+++
Cytokeratins in tumor sections	Positive	Positive	Positive	Positive	Positive

[a] The values of individual experiments are shown for ER and PR.
[b] IMH, immunohistochemistry.
[c] +, 1–25% of the cells are immunoreactive; ++, 25–50% of the cells are immunoreactive; +++, 50–75% of the cells are immunoreactive; ++++, 75–100% of the cells are immunoreactive.
[d] ND, not determined.

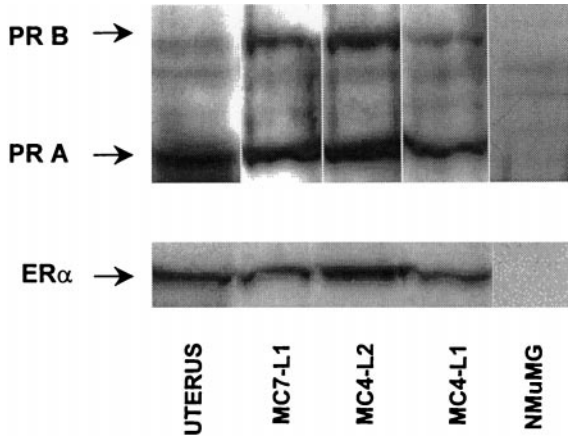

Fig. 4. Immunoblot analysis of PR isoforms (*PR A*, M_r 83,000; *PR B*, M_r 115,000) and ERα (M_r 66,000) in MC4-L1, MC4-L2, and MC7-L1 cell lines. Uteri from mice primed with 10 μg/kg E_2 and NMuMG cells were used as positive and negative controls, respectively. Cytosolic extracts were prepared as described in "Materials and Methods." Each sample (100 μg/lane) was separated on 7.5% (for PR) or 12% (for ER) SDS-PAGE, blotted to nitrocellulose, and probed with PR Ab 7. Signal was detected by a chemiluminescent reaction. Similar bands were obtained in the five cell lines using cells obtained from early (9–24) or late passages (41–96).

more variability than that observed in *in vitro* staining. This could be attributable to an intrinsic difference between tumors in the *in vivo* regulation of steroid hormone receptor expression, to the complex processing involved in paraffin embedding, in which it is more difficult to standardize all procedures as compared with *in vitro* cultures, or to a combination of both.

c-erbB2

Both membrane and cytoplasmic c-erbB2 staining ranging from moderate to high intensity was identified in all cell lines with the exception of MC7-L1, which gave only a mild reaction (not shown).

Effect of MPA and E_2 on Cell Proliferation

In primary cultures of the parental tumor line C4-HD, MPA had a strong stimulatory effect, exerting the highest stimulation at 10 nM ($P < 0.001$), whereas E_2 was inhibitory ($P < 0.01$; Fig. 5). At a similar range of concentrations, MPA slightly stimulated primary cultures of the C7-HI parental tumor line and E_2 also behaved as inhibitory ($P < 0.05$). In the cell lines, a different picture was observed. MPA was able to stimulate cell proliferation at a 10 nM concentration in all lines studied but only in experiments using early passages. After 20 passages, the polygonal cell lines MC4-L1, MC4-L3, and MC4-L5 became unresponsive or were stimulated only at concentrations of ~1 μM. The spindle-shaped cells (MC4-L2 and MC7-L1), however, were highly responsive at concentrations ranging from 1 nM to 1 μM, with the highest response at 1 μM (Fig. 6). E_2 also had a stimulatory effect in the spindle-shaped cell lines at concentra-

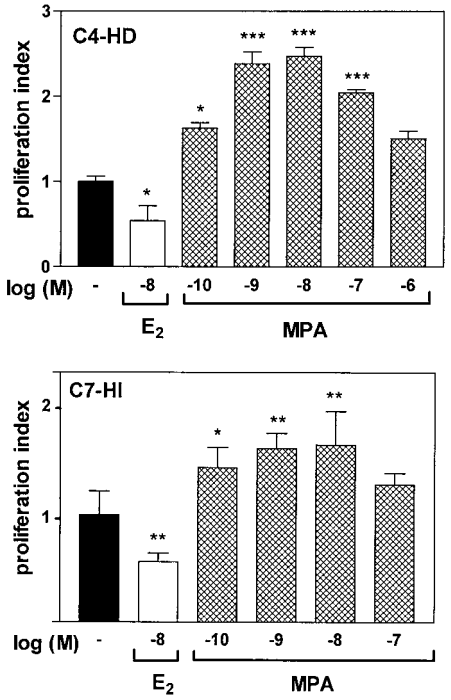

Fig. 5. Effect of MPA and E_2 on [^{3}H]thymidine uptake in C4-HD and C7-HI. The cells were seeded in 96-well microplates with 5% ssFCS; 24 h later, medium was replaced by experimental solutions (MPA, or E_2 in the presence of 1% ssFCS). Forty-eight h later, 50% of the media was replaced by fresh solutions, and 0.4 μCi of [^{3}H]thymidine was added to each well; 18–24 h later, the cells were harvested. A representative experiment of three is shown. The proliferation index is calculated as cpm of the experimental group/cpm control. *Bars*, SD. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; treated *versus* control.

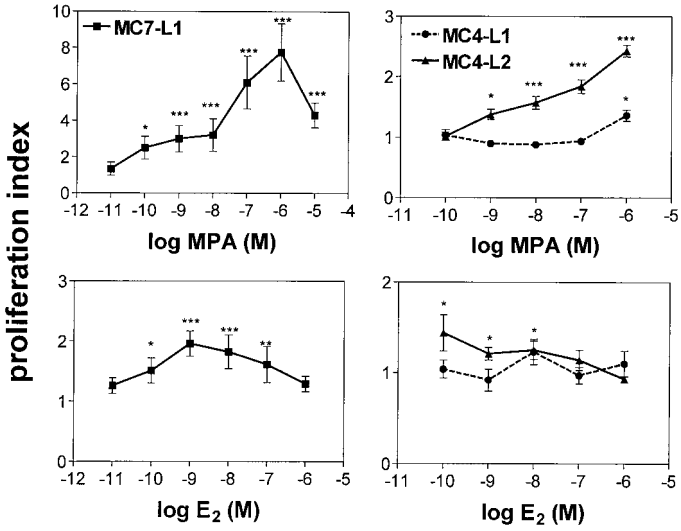

Fig. 6. Effect of MPA and E_2 on [^{3}H]thymidine uptake in MC7-L1, MC4-L1, and MC4-L2 in passages >20. The cells were seeded in 96-well microplates in the presence of 5% ssFCS. Twenty-four h later, the medium was replaced by experimental solutions (MPA or E_2 in the presence of 1% ssFCS). Forty-eight h later, 50% of the medium was replaced by fresh solutions, and 0.4 μCi of [^{3}H]thymidine was added to each well; 18–24 h later, the cells were harvested. A representative experiment of at least five experiments is shown. Proliferation index represents cpm of the experimental group/cpm control. *Bars*, SD. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, treated *versus* control.

tions lower than MPA (Fig. 6). Interestingly, the most progestin-responsive line originated from an *in vivo* progestin-independent tumor line.

Effect of MPA on *in Vivo* Growth

Cell lines from early passages were inoculated (10^6) s.c. in untreated or MPA-treated ovariectomized and control mice. Tumor take (latency, 1–2 months) was heterogeneous among groups; tumor growth was faster in MPA-treated mice. When these tumors were reinoculated in MPA-treated and untreated mice, a clearer picture was obtained; the polygonal cell lines showed a progestin-dependent pattern of tumor growth, whereas the spindle-shaped cells were progestin independent (Fig. 7). Curiously, these lines, which behaved *in vivo* as progestin independent, were those showing the highest response to estrogens and MPA *in vitro* (Table 3).

To further evaluate the behavior of the polygonal lines in later passages, MC4-L1 at passage 70 was inoculated in two MPA-treated and two untreated mice. Tumors grew similarly in both groups, but when a tumor growing in an MPA-treated mouse was transplanted again in MPA-treated and untreated mice, the progestin-dependent pattern of growth was reestablished (Fig. 7).

DISCUSSION

We report here the morphological and cytogenetic features, as well as the biological behavior, of five cell lines derived from MPA-induced progestin-dependent and progestin-independent murine mammary ductal carcinomas. To the best of our knowledge, there has been only one other description of a mouse mammary carcinoma cell line expressing steroid hormone receptors (31). This cell line was generated from a mammary carcinoma originated in transgenic mice overexpressing c-erbB2. In our experimental model, the tumors from which the lines were derived had been induced by MPA in female BALB/c mice in 1985 (22) and have since been maintained through syngeneic passages in hormone-treated (progestin-dependent) or untreated (progestin-independent) animals. The MPA-induced carcinomas are hormone-responsive metastatic ductal mammary tumors that express ERs and PRs (23). Their morphological and biological similarities with human ductal carcinomas, the most common malignant mammary tumors in humans (32), render these tumors a good model of breast cancer. In our model, progestins play a proliferative role, whereas in human breast cancer, estrogens are considered to be the main promoting/proliferative hormones; however, there is compelling evidence that progestins might also exert a proliferative effect (33, 34).

Four of the cell lines, MC4-L1, MC4-L2, MC4-L3, and MC4-L5, originated from the same progestin-dependent tumor (C4-HD); two, MC4-L1 and MC4-L2, were derived from the same primary culture, and the other two arose independently from different primary cultures. The fifth cell line, MC7-L1, originated from a progestin-independent tumor line (C7-HI). MC4-L1, MC4-L3, and MC4-L5 were similar, disclosing both *in vivo* and *in vitro* typical epithelial morphological features, whereas the other two (MC4-L2 and MC7-L1) showed a fusiform *in vitro* growth pattern and in syngeneic transplants disclosed either a biphasic appearance (MC4-L2) or the histological features of an anaplastic carcinoma (MC7-L1). Despite the different morphologies, all of the cell lines have proven to be of epithelial origin, as demonstrated by immunocytochemical studies using cytokeratins and E-cadherin. Electron microscopic examination confirmed these results and did not show the presence of viral particles.

PRs were detected by binding techniques, immunocytochemistry with two antibodies of different specificity, and by Western blots, where both A and B isoforms were identified. ERα was also detected by the same techniques, and we have not yet investigated the presence of ERβ. All assays were done using passages <20. The maintenance of these receptors was confirmed with Western blots using late passages (50–80). Spindle-shaped cells had higher receptor levels than epithelioid cells, as detected by binding techniques. Despite evident

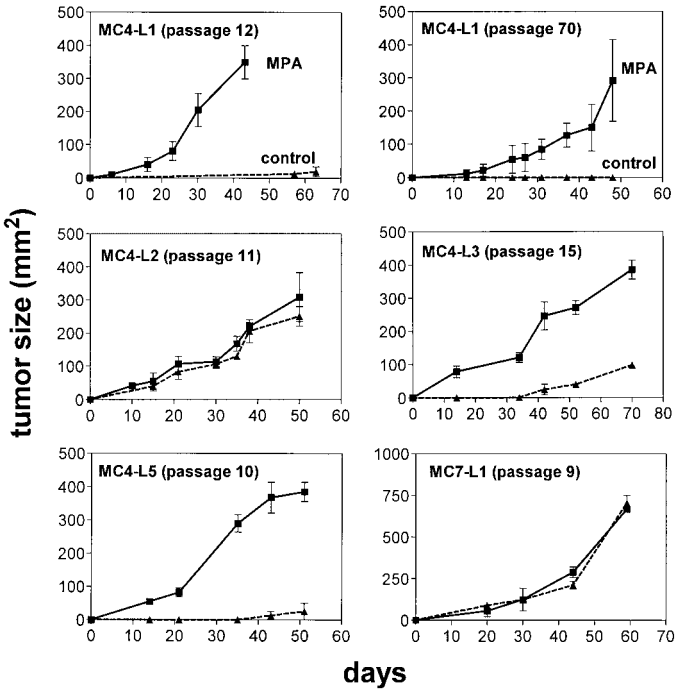

Fig. 7. Tumor growth of the different cell lines (passages 9–15) transplanted in MPA-treated and untreated mice. Cells (10^6) cells were s.c. inoculated in BALB/c female mice treated with MPA. Tumors were maintained by syngeneic transplantation in MPA-treated and untreated mice. Representative growth curves of *in vivo* passages 3–7 are shown. Tumor sizes were significantly different in MC4-L1, MC4-L3, and MC4-L5 lines throughout. To evaluate whether later passages of MC4-L1 maintain this pattern of hormone dependence, *in vitro* passage 70 was also studied. *Bars*, SD.

Table 3 *In vitro and in vivo hormone responsiveness of cell lines and their respective parental tumor lines*

Parental tumors	*In vivo*		Primary culture			*In vitro*		Inoculated cell line[a]
	MPA	E_2	MPA	E_2	Cell line	MPA	E_2	MPA
C4-HD	↑	↓	↑	↓	MC4-L1	−	−	↑
					MC4-L2	↑	↑	−
					MC4-L3	−	−	↑
					MC4-L5	−	−	↑
C7-HI	−	↓	↑	↓	MC7-L1	↑	↑	—

[a] Successive syngeneic passages in female BALB/c mice. −, no significant effect as compared with untreated controls; ↑, growth stimulation; ↓, growth inhibition.

variations in levels, all values were high enough to give an intense staining by immunocytochemistry or by Western blots.

All cell lines were tumorigenic in ovariectomized MPA-treated or untreated BALB/c mice. MC4-L2 and MC7-L1 tumors had similar growth curves in either MPA-treated or untreated animals, whereas the other MC4 lines grew significantly faster in MPA-treated as compared with untreated mice. All lines were metastatic, and MC4-L2 and MC7-L1, which showed distinctive *in vivo* and *in vitro* morphological features as compared with the other lines, were more aggressive when transplanted into syngeneic animals. The association in carcinomas of a change from epithelial to more mesenchymal features with an increased aggressiveness has already been documented (35, 36). Interestingly, the change from a typical epithelial morphology to the more "dedifferentiated" spindle cell or anaplastic features was not associated, in our tumors, with a significant modification of the pattern of expression of hormone receptors or hormone responsiveness, as described previously by others (37).

All lines displayed aneuploid karyotypes and disclosed common markers and many yet unidentified chromosomes that will be reported elsewhere. Aneuploidy is a characteristic of other murine mammary tumor cell lines (31, 38, 39).

Because c-erbB2 is overexpressed in most human breast cancers and its expression correlates with worse prognosis in several tumor types (40), we evaluated whether a similar correlation could be established in our cell lines. c-erbB2 was overexpressed in all MC4 lines, as described previously for parental C4-HD tumor (41). No overexpression was detected in the parental C7-HI tumor line, and immunoreactivity for c-erbB2 was weak in MC7-L1. No direct correlation was established between aggressiveness and c-erbB2 expression, because both lines are very aggressive *in vivo.*

The *in vivo*/*in vitro* differential hormone responsiveness is one of the most interesting features of the cell lines reported herein. MC7-L1, the most hormone-responsive line *in vitro*, was derived from an *in vivo* progestin-independent tumor line, and curiously, when these cells were re-inoculated in BALB/c mice, the autonomous growth pattern reappeared. The MC4 lines were derived from the *in vivo* progestin-dependent C4-HD tumor. In primary cultures, progestins also stimulated cell proliferation, whereas estrogens always played an inhibitory role. Surprisingly, three of the lines originated were hormone unresponsive *in vitro*, but they reacquired a hormone-responsive growth behavior when inoculated in BALB/c mice. Inversely, the fourth, MC4-L2, was stimulated *in vitro* by both estrogens and progestins but behaved as autonomous when inoculated in mice. Although we do not have a clear explanation for this switch in hormone responsiveness, the fact that estrogens had proven to be inhibitory in both parental tumors (26) and primary cultures (25), and now may exert proliferative effects in these cell lines, confirms that a careful evaluation must be carried out when extrapolating data from cell lines to primary tumors.

Strong evidence implicates estrogens in the development of human breast cancer. Most of the evidence is indirect and comes from: (*a*) epidemiological studies that have linked increased risk of developing mammary cancer to a high exposure to ovarian estrogens; (*b*) experimental models where estrogens have shown clear stimulatory effects in carcinogen-induced tumors (9, 10); (*c*) estrogen-responsive human cell lines (MCF-7, T47-D, and ZR-75–1); and, (*d*) the success of antiestrogen therapy as a first-line treatment in human breast cancer (42). There is also increasing evidence supporting a stimulatory role for progestins in mouse and in human normal and neoplastic mammary cells (34, 43). There is an increase in [^{3}H]thymidine uptake in normal mammary glands during the luteal phase of the menstrual cycle, coinciding with the peak of progesterone levels, and under certain experimental conditions, progesterone stimulates cell proliferation in several human cell lines (33). In different experimental models, progestins have also been used as inducers/promoters (22, 44, 45). It must be also pointed out that one of the most important physiological effects of estrogens is the induction of PRs (46). Finally, epidemiological studies linking increasing breast cancer incidence with high estrogen exposure do not rule out a similar role for high exposure to progestins. It has been reported recently that the addition of a progestin to hormone replacement therapy markedly enhances the risk of breast cancer relative to estrogen use alone (34).

On the other hand, both progestins and estrogens have also shown to exert inhibitory functions. MPA has inhibitory effects in breast cancer, and several reports suggest that this effect may not be mediated by PRs (47). Estrogens have also been used with success in the treatment of breast cancer (48) and have been shown to induce regression of a human breast cancer maintained by syngeneic transplantation in nude mice (49). Estrogens showed an inhibitory effect in ER-transfected cell lines (50). Also, Soto and Sonnenschein (51) have proposed that estrogens may exert inhibitory effects through shut-off mechanisms involving ERs. Antiestrogens have successively been used as a first-line cancer treatment, on the basis of their interference with the ER-mediated proliferative pathway. However, recent reports show that some of these antiestrogens may also act through other mechanisms, including the immune system (52). Moreover, the classical pure antiestrogen ICI 182,780 has been shown recently to act as an antiprogestin (53).

Our model provides experimental data showing that estrogens can behave as inhibitors or stimulators of cell proliferation and provide an interesting model to investigate the variables modulating this switch.

In synthesis, we report the establishment of a unique series of murine mammary carcinoma cell lines expressing ER and PR, which may be stimulated by progestins or estrogens. These lines are tumorigenic and metastatic in syngeneic mice, and they also show different patterns of hormone responsiveness *in vivo*. For these reasons, they provide an interesting experimental model for the study of hormone regulation in breast cancer.

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