Breast Cancer Research and Treatment **2807-03:** 1–12, 2003.

Report

Establishment of two hormone-responsive mouse mammary carcinoma cell lines derived from a metastatic mammary tumor

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Key words: antiprogestins, breast cancer, cell lines, estrogens, hormone dependence, hormone receptors, medroxyprogesterone acetate

Summary

We report the establishment of two mouse mammary cancer cell lines, MC7-2A and MC7-2B obtained from a mouse mammary carcinoma induced by medroxyprogesterone acetate (MPA) and maintained by syngeneic transplantation in BALB/c mice. They are epithelial (express cytokeratins) and express both estrogen receptors alpha (ERα) and progesterone receptors (PRs) isoforms A and B (western blots). *In vitro*, MPA inhibited ^{3}H-thymidine uptake, starting from concentrations as low as 10^{-13} M in MC7-2A and 10^{-10} M in MC7-2B; the antiprogestin RU 486 exerted a stimulatory effect at 10^{-14} M in both cell lines; 17-β-estradiol (E_2) also exerted a stimulatory effect starting at 10^{-10} M in MC7-2A and at 10^{-13} M in MC7-2B. When transplanted in syngeneic mice, both cell lines originated adenocarcinomas that gave rise to lung metastases within 3 months. In *in vivo* studies, in MC7-2A, the antiprogestin inhibited completely tumor growth, E_2 induced a slight although significant ($p < 0.05$) stimulatory effect and MPA stimulated tumor growth while MC7-2B cells were unresponsive to all treatments. ER and PR were also expressed in tumors as assessed by immunohistochemistry. Two marker chromosomes were identified by FISH as translocations between chromosomes 4 and 7, and between chromosomes X and 2; the third marker chromosome remains unidentified. All these markers were also present in the parental tumor. A new marker, a centric fusion of chromosomes 2, was acquired in both cell lines. Considering that there are very few murine breast carcinoma responsive cell lines, these cells represent new tools in which the regulatory effect of hormones can be studied.

Introduction

Many mouse mammary cell lines have been established, but only a few express the key growth regulatory proteins in breast cancer, estrogen (ER) and progesterone receptors (PRs). In fact, as far as we know there are only two models: our recently established hormone-responsive cell lines [1], which maintain the expression of ER and PR and show different degrees of hormone responsiveness; and more recently, the MXT+ and MXT [2].

We have developed a series of mouse mammary metastatic ductal carcinomas in female BALB/c virgin mice, using medroxyprogesterone acetate (MPA) as a carcinogen [3, 4]. These tumors express ER and PR and are progestin-dependent, growing only in progestin-treated mice, although we have been able to select hormone independent variants by successive transplantation in untreated animals. What makes this model unique is the fact that progestins have been shown to stimulate cell growth both *in vivo* and *in vitro* [5, 6], whereas antiprogestins and estrogens inhibit tumor growth [7, 8]. This model offers a specific system to study the proliferative effects of progestins and the inhibitory effects of estrogens, as well as the effects of the respective antihormones.

In a previous publication we described the features of five novel cell lines [1], four of them derived from

the progestin-dependent tumor line C4-HD and the other from a progestin independent tumor line, C7-HI. All lines expressed ER and PR and gave rise to mammary carcinomas when inoculated into syngeneic mice. In these lines, unlike what happens in the parental tumor, we found an inverse correlation regarding *in vivo* and *in vitro* hormone responsiveness. The lines that were unresponsive *in vitro* did respond *in vivo* and those lines which were unresponsive *in vitro* were responsive *in vivo*. Interestingly, 17-β-estradiol (E_2) that was inhibitory in parental tumors and in primary cultures exerted stimulatory effects on the cell lines.

The aim of our project was to develop mouse mammary cell lines that maintained the same hormone responsiveness as the parental tumors. The tumor line C7-2-HI was selected in this study because of its high metastatic ability, its stable hormone responsiveness, and because it has been used in previous studies regarding tumor regression [9]. In this study we report the characterization of two cell lines derived from C7-2-HI, named MC7-2A and MC7-2B.

Materials and methods

Hormones

MPA, mifepristone (RU 486) and 17-β-estradiol (E_2) were obtained from Sigma Chem. Co., St Louis, MO, USA, and were dissolved in absolute ethanol at 10^{-3} M (stock solution). Working solutions were freshly prepared before each experiment. MPA depot (Medrosterona, Lab. Gador, Buenos Aires, Argentina) and 5 mg E_2-silastic pellets were also used in *in vivo* experiments.

Steroid-stripped FCS (ssFCS)

To strip the sera of steroids, activated charcoal (Mallinckrodt Chemical Works, New York, NY, USA) was added to fetal calf serum (FCS) (Life Technologies Inc. [Gibco B.R.L.], Gaithersburg, MD, USA 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/F12 (Dulbecco's modified Eagle's medium: Ham's F12, 1:1, without phenol red [Sigma Chem. Co.]), 100 U/ml penicillin and 100 μg/ml Streptomycin. Growth medium: DMEM/F12 + 10% FCS (GM). Standard medium: DMEM/F12 + 5% ssFCS (SM).

Establishment of cell lines and culture conditions

A ductal MPA-induced progestin independent carcinoma, C7-2-HI [9], maintained by syngeneic transplantation, was used at passage 19.

Epithelial enriched cultures growing in the presence of SM and MPA were incubated in GM with the addition of penicillin 100 U/ml–Streptomycin 100 μg/ml after the first passage. After the third passage (approximately after 40 days), the cells became elongated and lost their epithelial morphology. In the fifth passage the cultures were subdivided into two lines that were henceforth maintained separately. Culture media was changed once a week and maintained for approximately 45 days without subculturing. Cells with epithelial morphology started to reappear in both cultures almost simultaneously as the growth rate of both cultures increased. In successive subcultures only the epithelial-like cells were selected and after a few passages no fibroblasts were observed. These cell lines were named MC7-2A and MC7-2B, and aliquots were routinely kept in liquid nitrogen to be used in further studies. Studies reported herein were performed using passages 14–34. Cells proved to be mycoplasm free.

Doubling time

Doubling time was evaluated by plating the cells in 6-well plates at a concentration of 20,000 cells/well and counting duplicate wells at 9 am and at 5 pm during 1 week. The values were calculated from the log phase of the growth curves.

Immunohistochemistry

Cell lines grown on culture flasks were washed in phosphate buffered saline (PBS) and fixed in formalin for 45 min for routine hematoxylin–eosin (H&E), or were detached and the suspension was centrifuged. In this case, the pellet was immersed in 20% gelatin in PBS. Once the gelatin had solidified, the preparation was fixed in 10% buffered formalin. Four micrometer of sections were obtained and were used for immunocytochemistry or were stained with H&E. Tumor tissue samples were frozen in liquid nitrogen or fixed in 10% buffered formalin (ER, PR) or ethanol (cytokeratins) and embedded in paraffin. Frozen

sections were obtained with a cryostat and fixed in formalin. 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 1:1000 dilution. ER (MC-20) and PR (C-20) rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA. These antibodies were diluted 1:100. Staining was developed with 0.06% 3,3′-diaminobenzidine (Sigma Co.). Selected slides were counterstained with hematoxylin or methyl-green. Negative controls were performed by replacing the primary antibody with normal rabbit serum.

Electron microscopy

Cell monolayers were detached from the flask with a rubber policeman, centrifuged, and fixed with 4% paraformaldehyde in PBS. Then, the cells were dehydrated, clarified with acetone 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

Semi-confluent 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 20 min at 37°C and the cells were fixed with 3:1 methanol–glacial acetic acid. The slides were stained with 3% Giemsa (Sigma Chem. Co.). The following passages were used: MC7-2A: 13 and 17 and MC7-2B: 15, 25 and 34. The chromosome number was expressed as the modal number, the number of chromosomes most frequently found after counting at least 100 metaphases. The metaphases were analyzed with G-banding [10] to determine numerical and structural abnormalities of the chromosomes. The chromosomes were identified on the basis of their banding pattern according to the Committee on Standardized Genetic Nomenclature for Mice [11].

Fluorescence in situ *hybridization (FISH)*

Mouse paint chromosome biotinylated DNA probes (Cambio, Cambridge, UK) were gently provided by Dr Marcelo Aldaz (Department of Carcinogenesis, The University of Texas M. D. Anderson Cancer Center, Smithville, TX). The metaphase chromosomes were denatured in 70% formamide/2X standard saline-citrate (SSC) (2 × SSC: 0.3 M NaCl, 0.03 M sodium citrate) at 70°C for 3 min and the probes were denatured at 70°C for 10 min. The probes were preannealed 50 min at 37°C. The hybridization was performed overnight at 37°C. After hybridization the slides were washed once in 2 × SSC, three times in 50% formamide/2 × SSC and three times in 0.1 × SSC, 5 min each time at 45°C. The probe was detected with fluorescein isothiocyanate (FITC) avidin (Vector Laboratories, Burlingame, CA, USA) and the chromosomes were counterstained with DAPI.

ER and PR

In vivo, the presence of ER and PR was evaluated by immunohistochemistry in tissue sections as described above, and *in vitro*, by ligand binding using the whole cell technique at single points [6] and by western blot using cell extracts [1].

Whole cell assay. 10^5 cells were plated in 24 well plates with GM. After 3 days, whole cell PR and ER assays were performed as previously described [1]. Briefly, a total of 300,000 cpm of 17-α-methyl ^{3}H-R5020 (NEN, Boston, MA, specific activity: 85 Ci/mmol) were added with a 100-fold excess of R5020 or ethanol, or 300,000 cpm of ^{3}H-E_2 (NEN, Boston, MA, specific activity: 86 Ci/mmol) together with 100-fold excess of DES 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 yielded 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 dithiothreitol, 20 mM Na_2MoO_4, 10% glycerol). Protease inhibitors (0.5 mM PMSF, 0.025 mM ZPCK, 0.0025 mM TLCK, 0.025 mM TPCK, 0.025 mM TAME) 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 [12].

Western blot analysis. Equal amounts of proteins (100 μg/lane) were separated on discontinuous 7.5% (PR) or 12% (ER) polyacrylamide gels [13]. A set of prestained M_r standards was run on each gel. Proteins were dissolved in sample buffer (6 mM Tris pH 6.8, 2% SDS, 0.002% bromophenolblue, 20% glycerol, 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 PBST 0.1% (0.8% NaCl, 0.02% KCl, 0.144% Na_2PO_4, 0.024% KH_2PO_4, pH 7.4, 0.1% Tween 20), washed several times with PBST and probed with PR Ab-7 (2 μg/ml, Neomarkers, Union City, CA) or ER MC-20 (1 μg/ml, Santa Cruz Biotechnology, CA) in PBST at room temperature for 2 h. The blots were washed three times, 10 min each, and probed with peroxidase-conjugated sheep anti-mouse Ig (PR) or peroxidase-conjugated donkey anti-rabbit Ig (ER) (Amersham Life Science, UK). The luminescent signal was visualized with the ECL western blotting detection reagent kit (Amersham International, PLC, UK), and exposed to a CURIX RP 1 (Medical X-ray film, Agfa). Western blots were performed using extracts of both cell lines. Uterus obtained from mice primed with E_2 10 μg/kg and NMuMG cells (gently provided by Dr JC Calvo, IBYME, Buenos Aires) were used as positive and negative controls, respectively. NMuMG cells are epithelial cells derived from mouse normal mammary gland [14].

Tumorigenicity

Cells were trypsinized and resuspended in 10-fold excess GM. After centrifugation, cells were resuspended in serum-free medium and 10^6 cells were injected subcutaneously (sc) in a final volume of 0.1 ml using a 21 gauge needle in the right inguinal flank of 2-month-old BALB/c female mice that had been inoculated contralaterally with 40 mg MPA depot ($n = 4$/group) or not. The mice were examined every 3 days. When tumor size was greater than 400 mm^2 the animals were sacrificed and a complete autopsy performed. These tumors were maintained by syngeneic passages in two untreated 2-month-old female BALB/c mice. Animal care was in agreement with the Guide for the Care and Use of Laboratory Animals [15].

Effect of MPA, RU 486 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 cells/ml. After attachment (24 h), the cells were incubated for 72 h with the experimental solutions to be tested (MPA, RU 486 and E_2: 1 fM–1 μM in 2.5% ssFCS). Fifty percent of the medium was replaced with fresh medium after 48 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 standard deviation were calculated for each solution tested.

Effects of MPA, RU 486 and E_2 on tumor growth

When tumors reached an approximate size of 40–60 mm^2, animals (4/group) were treated with either daily sc injections of saline solution (controls), 0.6 ml of RU 486 in saline solution (6 mg/kg), inoculated with 20 mg of MPA *depot* or implanted subcutaneously with 5 mg E_2-silastic *pellets*. Tumors were measured three times a week with a Vernier caliper. The experiments were repeated twice using passages 20 and 25.

Statistical analysis

The differences between controls and experimental groups in ^{3}H-thymidine uptake assays and tumor sizes in each day were analyzed by ANOVA followed by Tukey *t* test between groups. Tumor growth curves were also analyzed by regression analysis. Differences in receptor levels in cells were determined using Student *t* test. All the statistical analysis was performed using the GraphPad Prism™ (GraphPad Software, Inc., San Diego).

Results

Morphology

In vitro

The parental tumor C7-2-HI grew *in vitro* developing organoids with an ovoid center of polygonal malignant epithelial cells surrounded by an irregular crown of non-neoplastic fibroblasts. The growth patterns of

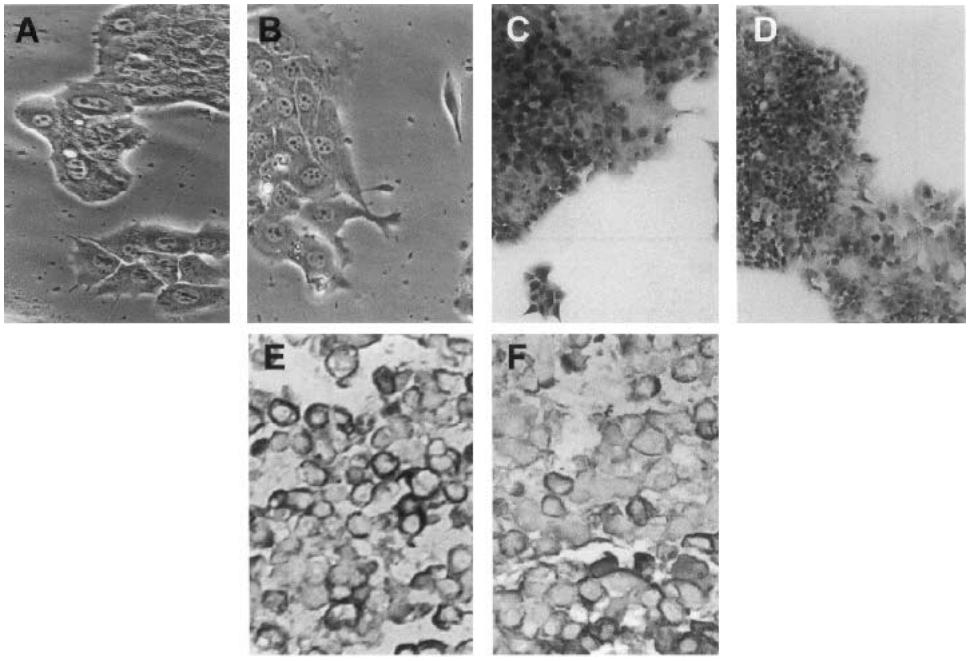

Figure 1. Phase Contrast Microscopy showing cohesive groups of polygonal cells with big nuclei and irregular nucleoli. Thin cytoplasmic filaments are evident; MC7-2A (A), MC7-2B (B) (200×). Hematoxylin and eosin staining showing several big clear cells intermingled with the more abundant polyhedral cells; MC7-2A (C), MC7-2B (D) (100×). Cytokeratin staining confirming their epithelial origin: MC7-2A (E), MC7-2B (F) (200×).

MC7-2A and MC7-2B were similar to that of their parental line (Figure 1(A)–(D)), except for the complete absence of fibroblasts. At low density they grew as attached monolayers but before reaching confluence, some cells would overgrow within the clusters, and tended to detach spontaneously. However, the adhesion of the cells remaining in the monolayer was high; several minutes of trypsinization were necessary for an efficient detachment. The epithelial nature of the cells was confirmed by immunocytochemical staining of cytokeratin (Figure 1(E) and (F)), and by electron microscopic studies. Both cell lines showed a similar ultrastructural pattern. Large intracytoplasmic vacuoles and membrane surface microvilli were detected in MC7-2A and MC7-2B (not shown). This last observation suggests that at least some cells are polarized *in vitro*. Electron microscopy also confirmed the absence of retroviral particles.

In vivo

MC7-2A. When inoculated sc in female BALB/c, MC7-2A cells originated moderately differentiated adenocarcinomas (Figure 2(A)). The cells grew as solid chords or groups of irregular tubular structures extensively infiltrating the surrounding stroma. Apoptotic figures were frequently encountered (Figure 2(A), inset). The neoplastic growth disclosed two cell populations, one polygonal in shape with clear, big vesicular nuclei, a round evident nucleoli and scant cytoplasms; the other with fusiform cells with abundant eosinophilic cytoplasms and dark, elongated nuclei. These elements were distinguishable from fibroblasts by their malignant cytological features, and because they expressed cytokeratins (Figure 2(C)). The fibroblastic stroma was, in areas, abundant and well vascularized and the tumor may be infiltrated by inflammatory cells. These tumors metastasized to lungs (Figure 2(E)), with a latency of 2–3 months.

MC7-2B. MC7-2B grew *in vivo* as a poorly differentiated adenocarcinoma, disclosing sheets of polygonal cells with scant cytoplasms. Glandular differentiation was only occasionally found (Figure 2(B)). Anisokariosis was evident and the nuclei showed clumped, coarsely granular chromatin with one or more eosinophilic nucleoli. A high number of mitosis per high power field were observed, in some areas more than 10 (Figure 2(B), inset). The stroma was scant and fibroblastic. The neoplasia was generally well-vascularized. Apoptotic images can be seen in

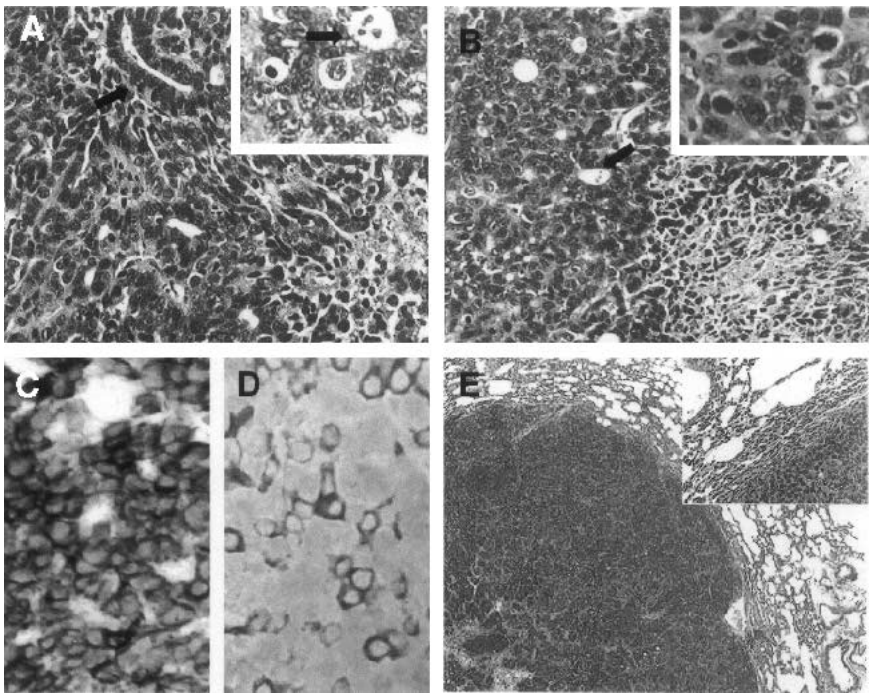

Figure 2. Morphological features of tumors. (A) MC7-2A: The tumor shows the structure of a ductal infiltrating carcinoma showing glandular differentiation (arrow) (100×). Necrotic areas and apoptosis (inset, 400×) are frequently encountered. (B) MC7-2B: This tumor is a less differentiated carcinoma although isolated glandular structures can be observed (arrow) (100×). Necrosis is evident in the right bottom corner. Several mitotic features can be observed in the inset (400×). (C) MC7-2A, cytokeratin staining (400×). (D) MC7-2B, cytokeratin staining (400×). (E) Lung metastasis of MC7-2A and MC7-2B (inset) (100×).

all the neoplastic tissue. MC7-2B also metastasized to lung (Figure 2(E), inset) with a latency of 3 months. The epithelial nature of MC7-2B was confirmed by immunohistochemical staining of cytokeratin (Figure 2(D)).

Doubling time

Both cell lines had similar doubling times. After a long lag phase of approximately 3 days, the doubling time of MC7-2A was 20.3 h (17.9–23.6) and 21.1 h (18.2–24.9) for MC7-2B.

Cytogenetics

Cytogenetic studies of the parental tumor line C7-2-HI revealed the presence of two populations: 85% of metaphases were nearly diploid with a modal chromosome number of 40–41, and 15% were hypotetraploid (range: 58–81 chromosomes). A representative karyotype is shown in Figure 3(A). Marker chromosomes were studied by G-banding and results confirmed by FISH. Three markers were found: M10, a translocation between chromosomes 4 and 7; M11, a translocation between chromosomes X and 2 (Figure 3(B)); and M12, which remains unidentified. Most of the metaphases of the tumor showed gains in chromosomes 2, 3, and 4, and losses of chromosome 8. Some meta-

Figure 3. (A) Representative G-banded karyotype of C7-2-HI diploid epithelial cells. M10: translocation between chromosomes 4 and 7; M11: translocation between chromosomes X and 2; M12: unknown. (B) Marker chromosomes M10 and M11 with G-bands and fluorescence *in situ* hybridization (FISH) with specific biotinylated probes to chromosomes 4 and 7 (M10) and to chromosomes X and 2 (M11); detected with FITC avidin in green. Chromosomes were counterstained with DAPI, in blue.

Figure 4. Representative G-banded karyotype of the cell lines. (A) MC7-2A. (B) Diploid population of MC7-2B. M10: translocation between chromosomes 4 and 7; M11: translocation between chromosomes X and 2; M12: unknown; M13: Robertsonian translocation between two chromosomes 2.

Figure 5. PR and ERα expression. (A) Immunohistochemistry in MC7-2A (frozen tissue, 200×) and MC7-2B (formalin fixed, 400×) tumors. (B) Western blots carried out using cell extracts. (C) Quantification by binding studies using the whole cell technique at single points. Data are expressed as media ± SD (fmoles/10^5 cells). Details of experimental procedures are described in Materials and method section.

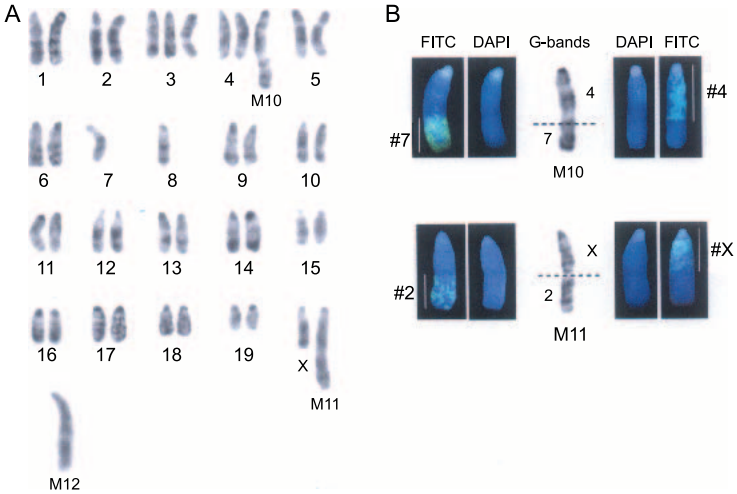

Figure 3.

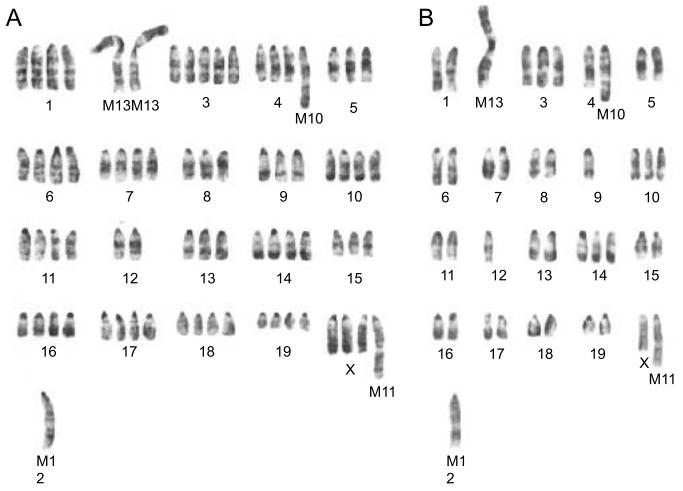

Figure 4.

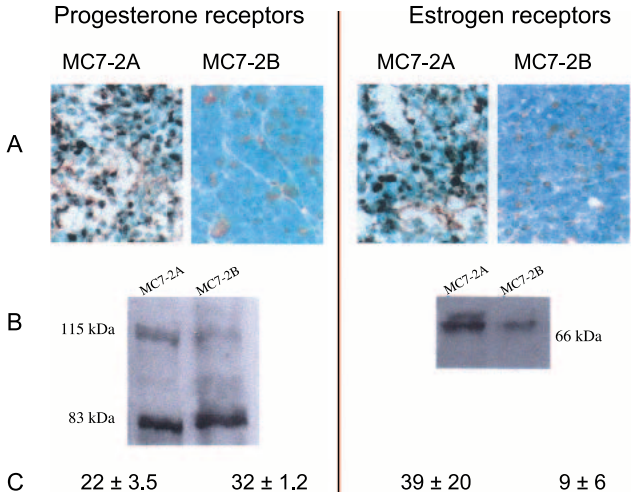

Figure 5.

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phases also showed monosomies of chromosomes 10, 12, 17, and 18, and trisomy of chromosome 7.

MC7-2A was constituted by a unique hypotetraploid population, with a modal number of 73 and a range of 58–78 chromosomes. G-band studies revealed the presence of the three markers observed in C7-2-HI (M10–M12) and an additional marker (M13), a Robertsonian translocation between two chromosomes 2. Chromosomes 2, 3 and 7 were over represented, and fewer copies of chromosomes 5, 8, 9, 12, 13 and 15 were observed (Figure 4(A)). Some metaphases showed losses of chromosome 10.

MC7-2B disclosed two populations, equally represented, one of a modal number of 41–42 with a range of 40–45 chromosomes, and the other with a modal number of 73 with a range of 66–82 chromosomes. Both populations showed the same four markers (M10–M13) observed in MC7-2A. Trisomies of chromosomes 2, 3, 7, 10 and 14, and monosomies of chromosomes 9 and 12 were observed in the diploid population (Figure 4(B)). Some diploid cells also showed monosomies of chromosomes 6, 8, and 13. The hypotetraploid cells presented a gain of chromosomes 2, 3, 7 and 11, and a loss of chromosomes 1, 5, and 6.

Hormone receptors

ER and PR were evaluated *in vitro* by binding techniques at a single point, by western blots and by immunohistochemistry in tumor tissues. A strong nuclear staining in the tissue sections was observed in both frozen (MC7-2A) or formalin fixed samples (MC7-2B) (Figure 5(A)). No staining was observed in negative controls. PR isoforms A (83 kDa) and B (115 kDa) and ERα (66 kDa) were detected by western blots (Figure 5(B)). The functionality of PR and ER was assessed in binding assays (Figure 5(C)).

Effect of hormones on cell proliferation

^{3}H-Thymidine uptake. In previous studies we demonstrated that MPA slightly increased cell proliferation in primary cultures of C7-2-HI [16] and that E_2 and antiprogestins exerted inhibitory effects (Figure 6). Under the same experimental conditions, early passages, (15–25) of MC7-2A and MC7-2B were inhibited even with pM concentrations of MPA (Figure 6). On the other hand, both RU 486 and E_2 exerted a stimulatory effect within the same range of concentrations (Figure 6). Hormone responsiveness was less evident in

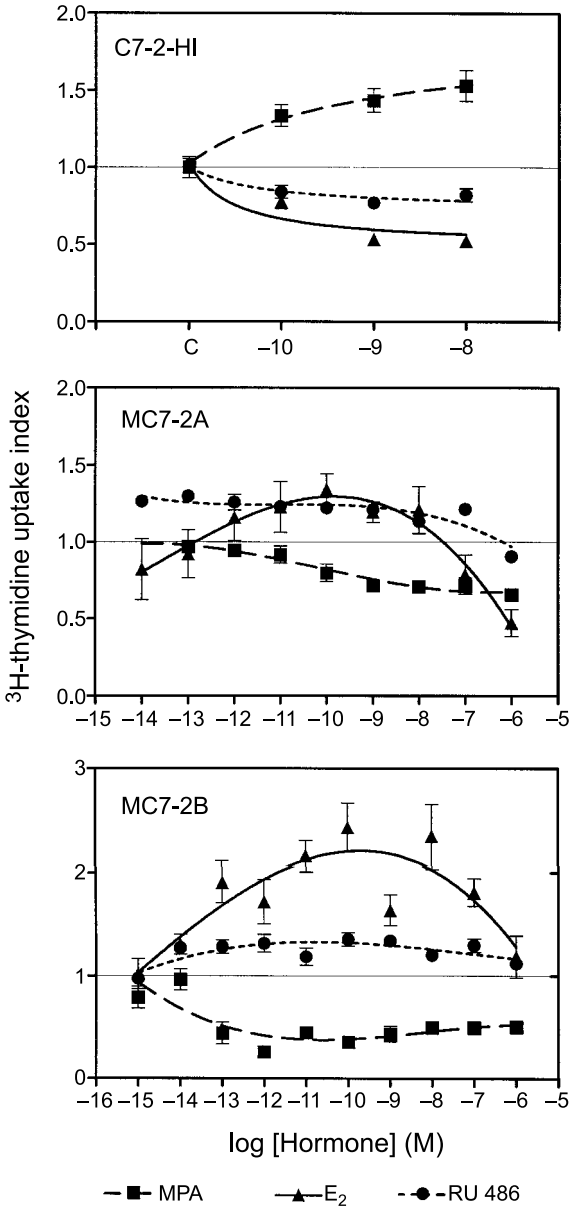

Figure 6. Effect of MPA, E_2 and RU 486 on ^{3}H-thymidine uptake in primary cultures of C7-2-HI, MC7-2A and MC7-2B in passages lower than 25. The cells were seeded in 96-well microplates in the presence of 5% ssFCS. Twenty four hours later the medium was replaced by experimental solutions (MPA, RU 486 or E_2 in the presence of 1% ssFCS. Forty eight hours 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. (MC7-2A) RU 486, $p < 0.01$ from 10^{-14} to 10^{-7} M; E_2, $p < 0.05$: 10^{-10} M and MPA, $p < 0.05$ from 10^{-10} M. (MC7-2B) RU 486, $p < 0.05$: 10^{-13} M, 10^{-12} M, 10^{-10} M, 10^{-9} M, and 10^{-7} M; E_2, $p < 0.05$ from 10^{-13} M to 10^{-7} M; MPA, $p < 0.001$: from 10^{-13} to 10^{-6} M.

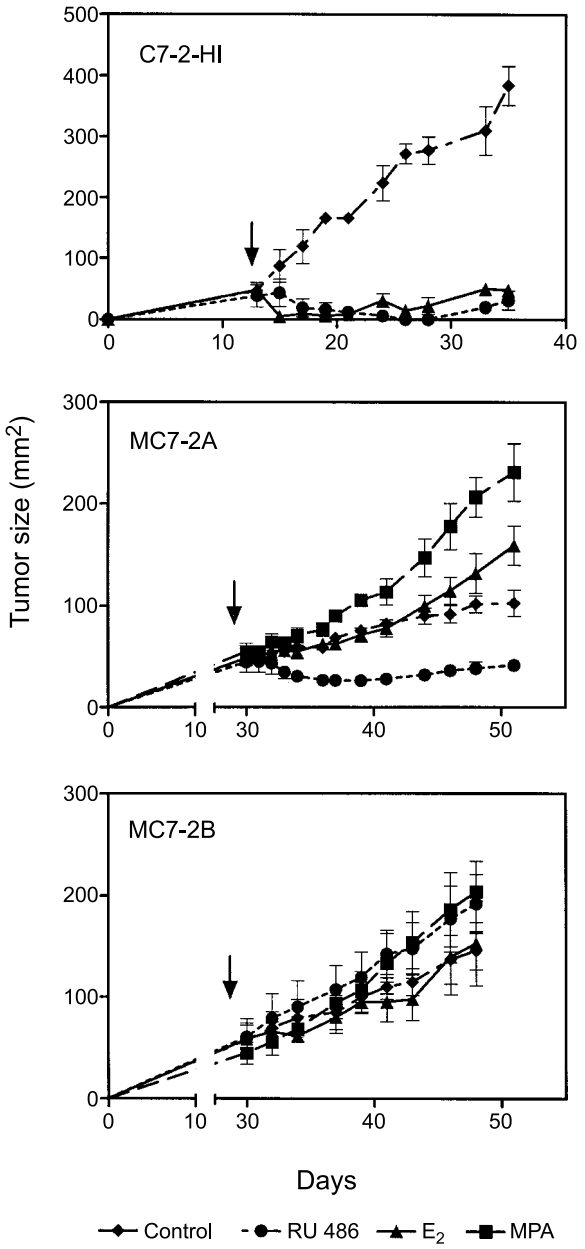

Figure 7. Effects of E_2 and RU 486 on *in vivo* growth of the parental tumor C7-2-HI and effects of MPA, E_2 and RU 486 on *in vivo* growth of MC7-2A (B) and MC7-2B (C). When tumors reached approximately 50 mm^2, the mice were administered daily with sc injections of RU 486 (6 mg/kg), saline (control), implanted with a subcutaneous pellet of E_2 (5 mg) or treated with 20 mg MPA depot sc (MC7-2A and MC7-2B). Four animals/group were used. The arrow indicates the time when the hormonal treatment begun. (MC7-2A) RU 486 treated group was significantly different from control starting from the second day ($p < 0.01$). E_2 treated group was significantly different only in the regression analysis ($p < 0.01$).

passages higher than 25, although the lines maintained ER and PR expression levels (not shown).

Tumorigenicity and effect of MPA, RU 486 and E_2 on in vivo *growth.* As previously described [9], C7-2-HI regresses after E_2 or RU 486 treatment (Figure 7). Mice treated or not with MPA were inoculated sc with 10^6 cells of either cell line. A representative experiment using passage 20 is shown. Tumor uptake was similar in both groups, thus tumors were transplanted to untreated mice to evaluate their *in vivo* hormone responsiveness. When tumors measured approximately 50 mm^2 hormone treatments were initiated. MPA increased MC7-2A tumor growth ($p < 0.05$), E_2 had a slight stimulatory effect ($p < 0.05$, linear regression analysis) and RU 486 was able to decrease tumor growth ($p < 0.01$) (Figure 7). No significant differences were observed with MC7-2B (Figure 7). The higher growth rate observed in the parental tumor may be due to the fact that it has been maintained by syngeneic transplantation for more than 30 passages. Cell lines inoculated *in vivo* usually grow more slowly because they need to adapt to *in vivo* conditions. After several *in vivo* passages they may grow even faster than parental cell lines. Anyhow, cells lines are routinely used in *in vivo* studies when inoculated directly in mice.

Discussion

In this paper we report the establishment of two novel mouse mammary carcinoma cell lines, MC7-2A and MC7-2B. The parental tumor C7-2-HI is an MPA-induced mammary carcinoma maintained by syngeneic transplantation which shares many common features with human breast cancer: ductal histology, autonomous tumor growth, expression of hormone receptors, responsiveness to endocrine therapy and metastatic behavior [9].

MC7-2A and MC7-2B differ from the previously described cell lines [1] mainly in their *in vitro* growth and hormone responsiveness. The latter grow as single cells which will eventually populate the entire flask surface as a monolayer, whereas the former develops clusters of highly packed epithelial cells. T47-D cells grow similarly at low density (personal observations). Regarding hormone responsiveness, this is the first time that an inhibitory effect for MPA in our experimental model was observed. E_2 and RU 486 on the other hand exerted stimulatory effects. After

passage 25 this hormone responsiveness was less evident, although steroid receptors were still expressed. It is noteworthy that concentrations as low as 100 fM were able to elicit increases (E_2 and RU) or decreases (MPA) in ^{3}H-thymidine uptake. This may be related to our recent description of a high affinity, low capacity-binding site (Kd: 43 ± 9 pM; $Q = 9 \pm 3$ fmol/mg prot.) in addition to the classical lower affinity, higher capacity-binding site (Kd: 9.2 ± 4.2 nM; $Q = 376 \pm 64$ fmol/mg prot.) for progesterone in MPA-induced mammary carcinomas [16]. Hypersensitivity to E_2 have already been described in different experimental settings in which cells respond to concentrations as low as 10^{-15} M [17–19], although the mechanisms involved still remain unknown.

In vivo, a different hormone response was observed: MPA stimulated, and RU 486 inhibited MC7-2A tumor growth. E_2 exerted a slight stimulatory effect. No significant effects were observed in MC7-2B tumors. These differences between *in vivo* and *in vitro* responses of cell lines to hormones highlight the need of *in vitro* experimental models involving stromal tissue.

Although both cell lines were indistinguishable *in vitro*, *in vivo*, MC7-2B was more anaplastic and hormone unresponsive. Curiously this cell line also showed two cell populations equally represented: a nearly diploid and a hypotetraploid population. MC7-2A showed only the hypotetraploid population, although was more hormone-responsive, suggesting that hormone resistance is not associated with increases in ploidy.

Differences between *in vitro* and *in vivo* responses to hormones have already been reported in the MC4 cell lines [1]. Primary cultures of the parental tumor C7-2-HI also showed a different response as compared with MC7-2A and MC7-2B: MPA slightly stimulated while RU 486 and E_2 slightly inhibited cell proliferation. *In vivo*, the latter induced nearly complete C7-2-HI tumor regressions [9].

Progestins have been shown to exert inhibitory effects in several human breast cancer cell lines such as ZR-75-1, MDA-MB-453, T-47D, and MCF-7 under certain experimental conditions [20–22]. Increases in cell proliferation have also been reported in the same cell lines, especially in T-47D [23]. The mechanisms by which MPA modulates cell proliferation are still unknown. Some authors suggest that androgen receptors may be mediating MPA's inhibitory effect [20, 24, 25], while others point towards a PR mediated effect [21, 22]. In PR-transfected MDA-MB-231 cells, progesterone has shown to exert inhibitory effects [26], although this seems to be a common feature in steroid receptor transfected systems since E_2 also inhibits cell proliferation in ER transfected MDA-MB-231 cells [27].

Antiproliferative effects of antiprogestins as RU 486 have also been described [30]. RU 486 was found to have antiproliferative effects in the same lines and at the same experimental conditions as MPA [28–30], making the interpretation of both results more difficult. In our cells, progestins and antiprogestins have had always the opposite effect, suggesting that their actions are specific and mediated by PR.

There are very few models in which the hormone responsiveness of the cell lines can be compared with that of the parental tumor. In human breast cancer is nearly impossible. No other cell lines expressing ER and PR are available in mice in addition to our previous reported cell lines and the MXT model. In our previous cell lines we have demonstrated that while E_2 was inhibitory in the parental tumor, in both *in vivo* and *in vitro* studies, cell lines were either unresponsive or they were stimulated by E_2. In the MXT cell lines, no reference has been made to their ability to respond to either E_2 or MPA, only their response to high tamoxifen concentrations is mentioned (higher than micromolar) [2].

In rats, although there are several breast cancer cell lines established, a recent study reports on the characterization of the first breast cancer cell line derived from a methylcholantrene induced tumor model in Fisher rats, maintained by transplantation, which shows hormone responsiveness both *in vivo* and *in vitro* [31]. It has been suggested that the lack of hormone responsiveness in rat cell lines was mainly due to inappropriate experimental procedures [31]. These problems were overcome if experiments were performed using high serum concentrations confirming Sonnenschein and Soto's hypothesis regarding estrogens binding to serum born inhibitors [32]. In our experiments we used only low (1–2.5%) steroid stripped serum concentrations.

We postulate that in the process of cell line establishment several changes occur within cells. Before a neat population of cell arises, the cultures are characterized by the presence of an important chaos of intermingled multinucleated cells mixed with stromal-like cells. Within this context it seems that certain cells acquire new characteristics which confer advantages to grow in the absence of other regulatory signals and give immortality in plastic. This hypothesis is sustained by the fact that in our lab, from 8-cell lines developed including these cell lines, all acquired

new marker chromosomes, or has duplicated parental markers. Results obtained in cell lines regarding hormone responsiveness are probably not extended to parental tumors. Considering that most of the data regarding the proliferative effects of hormones in breast cancer have been obtained using breast cancer cell lines, the role of estrogens and progestins on breast tumor growth should be revisited. Differences between tumors and cell lines derived from those tumors have been reported also by others [33, 34].

Marker chromosomes of C7-2-HI were different from those observed in C4-HD [35], a ductal progestin-dependent mammary carcinoma also induced by MPA. However, chromosomes 2 and 4 were involved in markers in both tumors. Trisomies or over representation of chromosomes 3 and 4 and monosomies of chromosomes 9 and 13 were also common features of both MPA-induced carcinomas. Chromosome 9 has been considered to contain candidate tumor suppressor genes in other studies [36]. The relevance of these findings in MPA-induced carcinogenesis will be revealed after the screening of a larger number of tumors.

In summary, we have developed and characterized two hormone-responsive mouse mammary carcinoma cell lines expressing estrogen and progesterone receptors which, when transplanted *in vivo*, originate ductal metastatic carcinomas. These lines provide an innovative tool to study the mechanisms of hormone responsiveness and hormone-regulated tumor growth.

Acknowledgements

We are grateful to Laboratorios Gador, Buenos Aires, Argentina, for supplying MPA, to Julieta Bolado and María Alicia Gorostiaga for excellent technical assistance and to Dr N. Sanjuan for electron microscopic studies. This work was supported by Fundación Sales (Specific Grant 2000–2002) and SECYT (BID 1201/OC-AR, PICT 99, 05-06389).

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