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PRECLINICAL STUDY

Establishment of an in vitro estrogen-dependent mouse mammary tumor model: a new tool to understand estrogen responsiveness and development of tamoxifen resistance in the context of stromal–epithelial interactions

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Abstract Currently, to our knowledge, there are no continuous cell lines derived from estrogen dependent, tamoxifen sensitive spontaneous mouse mammary carcinomas. We describe here the establishment and characterization of a cell line derived from the M05 mouse mammary tumor, LM05-Mix, composed of both an epithelial and a fibroblastic component. From it the respective epithelial LM05-E and fibroblastic LM05-F cell lines were generated by limiting dilution. Immunofluorescence studies confirmed that the epithelial cells were positive for E-cadherin, cytokeratins and vimentin whereas the fibroblastic cells were negative for the epithelial markers and positive for α-smooth muscle actin and vimentin. Both cell types expressed estrogen and progesterone receptors, although only the epithelial LM05-E cells were stimulated by estradiol and inhibited by tamoxifen. In the bicellular LM05-Mix cell line estradiol proved to stimulate cell proliferation whereas the response to tamoxifen was dependent on confluency and the degree of epithelial-fibroblastic interactions. The presence of membrane estrogen receptors in both cell types was suggested by the achievement of non-genomic responses to short treatments with estradiol, leading to the phosphorylation of ERK1/2. Finally, cytogenetic studies suggest that these two cell types represent independent cell populations within the tumor and would not be the result of an epithelial-mesenchymal transition. This model presents itself as a valuable alternative for the study of estrogen responsiveness and tamoxifen resistance in the context of epithelial-stromal interactions.

Keywords Breast cancer · Estrogen receptor · In vitro mouse model · Tamoxifen

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Introduction

Breast cancer is the most frequent cancer in women in industrialized countries [10] (22% of all cancers) and is the second leading cause of cancer death [7]. Estrogens, in particular, have long been associated with the pathogenesis of this disease, playing a key role in sustaining the growth of breast cancer cells that express the receptor for this hormone [11]. Tamoxifen has been the main hormonal therapy for both early and advanced breast cancer patients for approximately three decades. As a matter of fact, tamoxifen was the first target-based agent directed against a growth-promoting pathway that entered clinical practice. Approximately 50% of patients with advanced disease do not respond to first-line treatment with tamoxifen. Furthermore, almost all patients with metastatic disease and approximately 40% of the patients that receive tamoxifen as adjuvant therapy experience tumor relapse and die from their disease. These findings strongly suggest that mechanisms of de novo or acquired resistance to tamoxifen occur

in breast cancer patients, and that this phenomenon might largely affect the efficacy of this treatment. As such, the development of clinically relevant models to study the biology of hormone-refractory breast cancer is of great interest.

Established human cell lines have been the main source of knowledge for understanding the biology of estrogen responsive breast cancer. In particular the MCF-7 [14], T-47D [6] and ZR-75–1 [3] have been the most widely used. They all express estrogen and progesterone receptors and are hormone-responsive. However, as most cell lines, they only represent one subpopulation of the tumor parenchyma and as such do not allow the study of the interactions between different cell types within the tumor. As to mouse models, only two have been used to understand the biology of hormone-dependent breast cancer: the MXT model, which was induced by urethane and is estrogen dependent [17], and the medroxyprogesterone-acetate induced mouse mammary tumors which express ER and PR but that are progesterone dependent [8]. In both cases continuous cell lines have been established as in vitro models [1, 9].

We recently reported the establishment of an estrogen dependent, tamoxifen sensitive in vivo mouse mammary cancer model which we called M05 [13]. We report here the characterization of the LM05-Mix continuous cell line derived from the M05 tumor, which is composed of an epithelial and a fibroblastic cell population. From it we generated the epithelial LM05-E and fibroblastic LM05-F cell lines. Our results show that both cell types express estrogen and progesterone receptors which are able to modulate the proliferation and cell death in a cell-type specific manner and that fibroblastic–epithelial interactions modulate these responses. On the other hand the presence of functional membrane receptors is implied by rapid non-genomic responses to estrogen. Finally, cytogenetic studies suggest that the LM05-E and -F cell lines were not originated as result of a transition from one to the other. Thus, these cell lines may prove to be a valuable tool for the study of hormone-responsiveness in the context of stromal–epithelial interactions.

Materials and methods

Tumor

The M05 mouse mammary tumor appeared spontaneously in a virgin BALB/c female mouse in our breeding colony. It was diagnosed as a semi-differentiated adenocarcinoma with areas of papillary differentiation. Since then it has been maintained by subcutaneous serial transplantation to the flanks of virgin female syngeneic mice [13].

Cell culture

From a passage 6 M05 tumor, a primary cell suspension was obtained by enzymatic digestion with 0.01% Pronase (Sigma, St Louis, MO) and 0.0035% DNAse (Sigma) in DMEM/F12 medium (Sigma). Two cell populations were identified within the culture, which was thus named LM05-Mix. At passage 8 the two cell populations were separated and cloned by limiting dilution to generate the LM05-E and -F clones. All three cell lines have since been stable in morphology for at least 40 passages. The LM05 cell lines were routinely maintained in DMEM/F12 medium (Sigma), supplemented with 10% fetal calf serum (FCS, GenSA, Buenos Aires, Argentina), in a humidified 5% CO_2/air atmosphere. Serial passages were carried out by treatment of 80% confluent monolayers with 0.25% trypsin and 0.02% EDTA in Ca^{2+}-free and Mg^{2+}-free PBS.

Proliferation assays

One hundred thousand cells were seeded in 35 mm dishes in DMEM/F12 medium containing 10% FCS. The following day dishes were washed twice with PBS and medium was replaced by DMEM/F12 without phenol red (Sigma) containing 1% charcoal stripped FCS. Cells were treated with either 17-β-estradiol (Sigma), medroxiprogesterone acetate (Sigma), 4-OH-tamoxifen (Sigma) or the anti progestin RU486 (Sigma) which were all previously prepared as stock solutions (1,000×) in absolute ethanol. At different time points, cells were washed twice with PBS, harvested and counted using a Neubauer chamber. Experiments were carried out in triplicate, and were repeated at least three times. Controls were treated with the corresponding dilution of absolute ethanol.

Immunofluorescence

Cells grown on glass coverslips or Lab-Tek assemblies were fixed in 4% formalin in PBS for 20 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS at 37°C for 20 additional minutes. Non-specific sites were blocked with 2% FCS in PBS for 1 h at room temperature. Primary antibodies were prepared in blocking buffer and applied overnight at 4°C in a humidified chamber. Samples were washed in PBS and incubated with secondary antibodies prepared in blocking buffer for 1 h at room temperature. The slides were then washed and counterstained with propidium iodide (Sigma) or 4′6-diamino-2-phenylindole (DAPI Research Organics, Inc.) and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Samples were analyzed under a Nikon Laser Confocal Microscope (Nikon, Tokyo,

Japan) or a Nikon fluorescence biological microscope (Eclipse E400).

Western immunoblot

Extracts were prepared by homogenizing cells on ice in RIPA buffer (50 mM Tris, pH 8.0 with 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate and 1% NP40) containing protease inhibitors (40 μm phenylmethylsulfonyl fluoride, 5 μg/ml leupeptina, 50 μg/ml aprotinin and 200 μM orthovanadate). Protein concentrations were measured using the Bradford method. Samples were mixed with 4× sample buffer containing β-mercaptoethanol and boiled for 2 min. Fifty micrograms of each sample were then separated in 6 and 8% SDS-PAGE mini gels (BioRad) and transferred to PVDF membranes (Amersham Biosciences, Uppsala, Sweden). The membranes were blocked overnight in 5% fat free milk and 0.1% Tween-20 in PBS (PBST) at 4°C. Primary antibodies were used at a 1/500 dilution in PBST with 2.5% fat free milk, and were incubated at 4°C overnight. After washing with PBST, membranes were incubated with secondary antibodies at a 1/1,000 dilution for 1 h at room temperature. Signals were detected with an enhanced chemiluminescence kit (ECL, Amersham Biosciences).

Antibodies

The following primary antibodies were used: rabbit anti-E-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-smooth muscle actin (Sigma), rabbit anti-progesterone receptor (Santa Cruz Biotechnology), rabbit anti-estrogen receptor-alpha (Santa Cruz Biotechnology), goat anti-estrogen receptor-beta (Santa Cruz Biotechnology), sheep anti-estrogen receptor-beta (a kind gift of Dr. Gustafffson), mouse anti-progesterone receptor (Novocatra, Newcastle, UK), rabbit anti-bovine cytokeratins (a kind gift of Dr. Mina Bissell) rabbit anti-vimentin (Abcam), rabbit anti-p44/42 Map kinase (Cell Signaling) and rabbit anti-phospho p44/42 Map kinase (Cell Signaling). For immunofluorescence the following secondary antibodies were used: FITC-conjugated goat anti-rabbit (Zymed, San Francisco, CA), FITC-conjugated rabbit anti-goat (Pierce Biotechnology, a kind gift of Dr Mary Helen Barcellos-Hoff), and FITC-conjugated rabbit anti-mouse (Zymed). For western blots the following peroxidase conjugated secondary antibodies were used: goat anti-rabbit (Amersham), and rabbit anti-mouse (Amersham).

Cytogenetics

Cells in culture were treated with 0.5 μg/ml colcemid (Life Technologies Inc.)/colchicine (Sigma) for 2 hours at 37°C, and detached with 0.25% trypsin. Hypotonic treatment was performed in 0.075 M potassium chloride for 20 min at 37°C and the cells were fixed with cold 3:1 methanol-glacial acetic acid. The metaphases were analyzed using G-banding and C-banding. The chromosome number was expressed as the modal number, the number of chromosomes most frequently found after analyzing at least 50 metaphases, and chromosomes were identified on the basis of their banding pattern according to the Committee on Standardized Genetic Nomenclature for Mice [2].

Fluorescence in situ hybridization

Mouse paint chromosome biotinylated DNA probes (Cambio, Cambridge, UK) were used. The metaphase chromosomes were denatured in 70% formamide/2× 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 pre-annealed 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 probes were detected with fluorescein isothiocyanate (FITC)-avidin (Vector Laboratories, Burlingame, CA, USA) and the chromosomes were counterstained with Propidium Iodide. The signal was viewed in a Nikon eclipse confocal microscope.

Apoptosis

Apoptosis was assayed in cell cultures using a commercially available kit (In Situ Cell Death Detection Kit, Flourescein, Roche Applied Science, Bromma, Sweden), designed to detect terminal deoxynucleotidyl transferase (TdT)-mediated nick end labeling (TUNEL). Cells were fixed in 4% formalin and permeabilized in cold 0.1% Triton X-100 in 0.1% sodium citrate. After washing with PBS, cells were incubated in TUNEL reaction mixture at 37°C for 60 min, washed, counterstained with DAPI and mounted.

RT-PCR

RT-PCR RNA was isolated from LM05 E, F and Mix cell lines using TRIZOL reagent (Invitrogen) in accordance with the manufacturer's protocols. The cDNA was made with the iScript tm cDNA Synthesis Kit (Biorad) following the manufacturer's protocol, with an initial amount of RNA of 1 μg. The ER-β cDNA was amplified using the following sense and antisense primers 5′-ATTGCCTGA ACAAAGCCAAG-3′ and 5′-GCCAGTGAGGGTCTTCT GAG-3′.

Statistical analysis

The statistical significance of differences between groups was calculated by applying Student's *t*-test or ANOVA. A value of $P \leq 0.05$ was considered significant.

Results

The LM05-Mix cell line is composed of an epithelial and a fibroblastic cell population

From a primary culture of the estrogen dependent, tamoxifen sensitive M05 mouse mammary tumor, the LM05-Mix continuous cell line was established. After eight passages the LM05-Mix cell line achieved a stable morphology which, by observation under phase contrast microscopy, revealed the presence of two cell populations: islets of small cobblestone-like epitheloid cells surrounded by loosely arranged large and clear spindle cells (Fig. 1a). Each of these subpopulations was separated by limiting dilution giving rise to the LM05-E and LM05-F cell lines, respectively (Fig. 1a). To determine the lineage of these cells we carried out immunofluorescence studies. The epithelioid cells were positive for E-cadherin, vimentin and cytokeratins (Fig. 1b). On the other hand the fibroblast-like cells were negative for cytokeratins and E-cadherin and positive for α-smooth muscle actin and vimentin (Fig. 1b). The clones showed the same pattern of staining as did each of the respective subpopulations in the LM05-Mix cell culture (data not shown).

The LM05 cell lines express estrogen and progesterone receptors and are hormone-responsive

Given that the LM05 cell lines derive from an estrogen-dependent mouse mammary tumor that expresses estrogen and progesterone receptors [13], we next investigated whether hormone receptors were present. Immunofluorescence and western blot studies revealed the presence of ER-α and PR in the LM05-Mix, -E and -F cell lines (Fig. 2a, b, respectively). On the other hand ER-β was detected at a lower intensity by immunofluorescence (Fig. 2a), and RT-PCR studies confirmed the presence of expression at the mRNA level in the three cell lines (Fig. 2c).

Next, to determine the hormone-responsiveness of the cells we carried out proliferation assays in the presence of 1% charcoal-stripped FCS. We observed that estradiol 10^{-8} M had a statistically significant stimulatory effect on the LM05-E cells while tamoxifen 10^{-6} M had an inhibitory effect (Fig. 3a), with the appearance of dead cells as observed under the phase contrast microscope. Similar results were obtained on the LM05-Mix cell line only when treatments were initiated at low confluency (Fig. 3b). However, when experiments were started at a higher degree of confluency the inhibitory effect of tamoxifen was greatly reduced (Fig. 3c), and cell death was not evident.

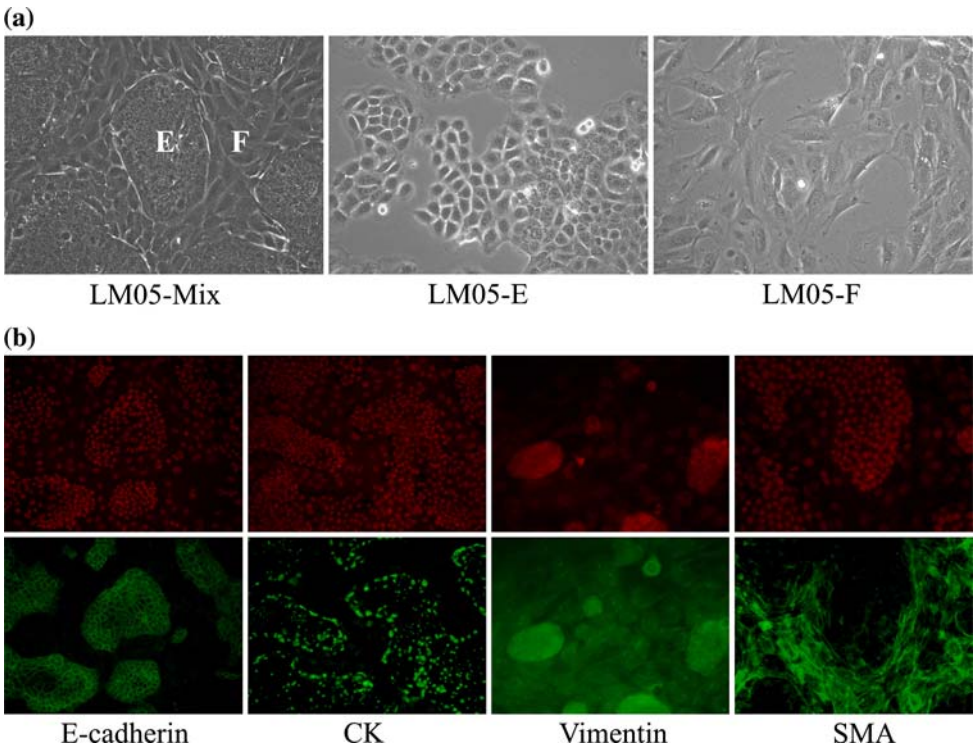

Fig. 1 Characterization of the LM05 cell lines. (**a**) Appearance of the LM05 cell lines under the phase contrast microscopy. The left panel shows the LM05-mix cell line where two cell types can be clearly distinguished; cobblestone like epithelial cells (E) organized in islets are surrounded by larger stromal like cells (F) that appear in a stream-like organization. (**b**) Immunofluorescent characterization of the LM05-Mix cell line. The epithelial cells were positive for E-cadherin, cytokeratin (CK) and vimentin, whereas stromal cell were positive for α-smooth muscle actin (SMA) and vimentin. In all cases the top panel shows the nuclei stained with propidium iodide (red) and the cell markers with FITC (green). Magnification 400×

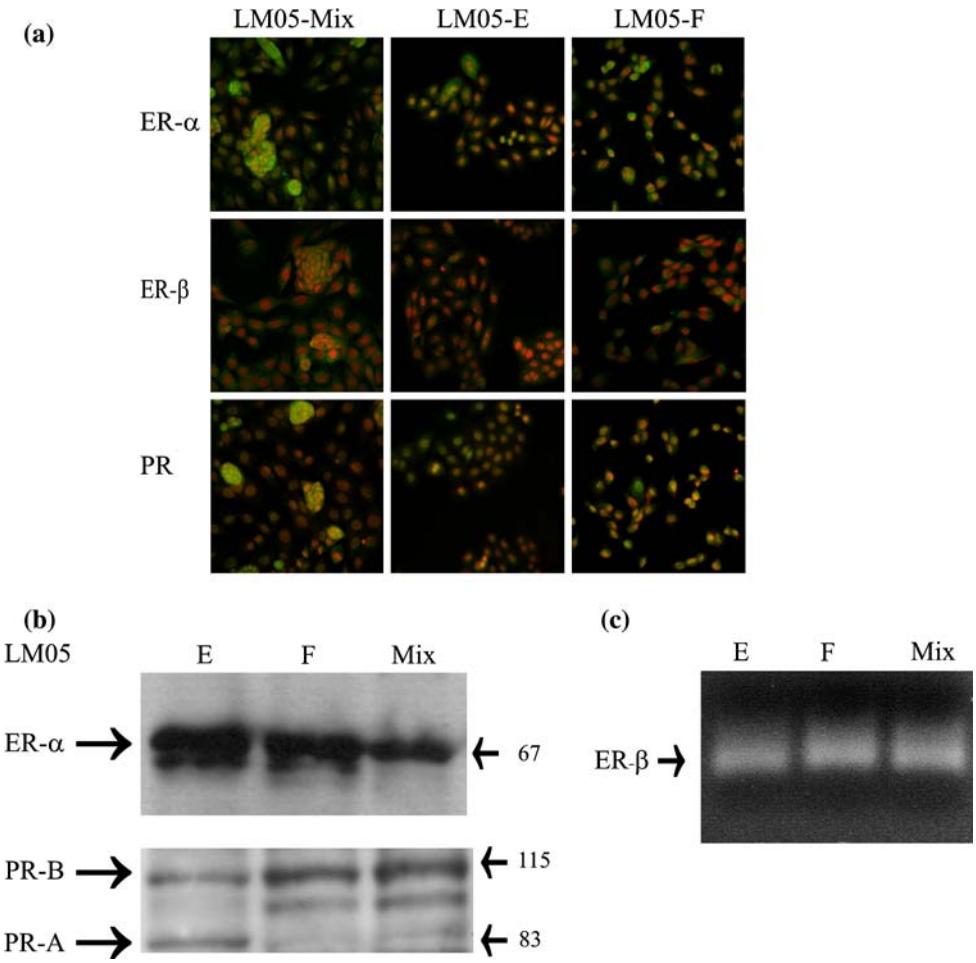

Fig. 2 Expression of estrogen and progesterone receptors in the LM05 cell lines. (**a**) Immunofluorescent staining to estrogen and progesterone receptors showed that the three cell lines were positive for ER and PR. In all cases the confocal images show steroid receptors stained with FITC (green) and the nuclei counterstained with propidium iodide (red). Magnification 400×. (**b**) Western blots to ER-α and PR confirm the presence of the receptors in the three cell lines. (**c**) Confirmation of the presence of mRNA to ER-β in the three cell lines by RT-PCR. The band corresponds to the expected molecular weight of the RT-PCR product

Interestingly, proliferation of the LM05-F cell line was not significantly modulated by either estradiol or tamoxifen (Fig. 3d).

Having observed a differential effect of tamoxifen on cell death that seemed to be dependent on the establishment of cell–cell interactions only on the LM05-Mix cell line, Tunel assays were carried out on 80% confluent LM05-E and LM05-Mix cells in the presence and absence of tamoxifen. As expected an increase in the number of apoptotic cells was only observed in the LM05-E cells treated with tamoxifen, but not in the LM05-Mix cell line (Fig. 4a, b), suggesting that stromal–epithelial interactions could be mediating tamoxifen resistance.

Estrogen receptors have been shown to carry out their effects not only as transcription factors, but at the cell membrane exerting rapid non-genomic signaling in the presence of ligand. To investigate whether the LM05 cells responded in this way to estrogens, starved LM05-Mix, -E and -F cells were treated for different periods of time with 10^{-8} M estradiol and activation of the MAPK ERK1/2 pathway was evaluated by western blot. As shown in Fig. 5 the three cell lines responded to estradiol within 5–10 min of treatment, revealing the presence of functional membrane ER. Interestingly, the LM05-F cell line, which did not respond to estradiol with an increase in cell proliferation, responded by activation of the MAPK pathway, suggesting that ER may be involved in other aspects of the biology of these stromal cells that are not directly linked to cell proliferation.

Cytogenetics

Mammary epithelial tumor cells have been proposed as to being able to give rise to tumor stroma; therefore to determine whether the LM05-E and LM05-F cell populations were related we carried out cytogenetic studies. The study of the metaphases from the LMO5-E cell line revealed a modal number of 76 chromosomes, with all the metaphases distributed in a range of 70–81 chromosomes for passage 12. The LMO5-F line showed a modal number of 126–129 chromosomes and a range of 116–129 chromosomes for the passage 13, and a modal number of 124, with a range of 94–126 chromosomes for the passage 17.

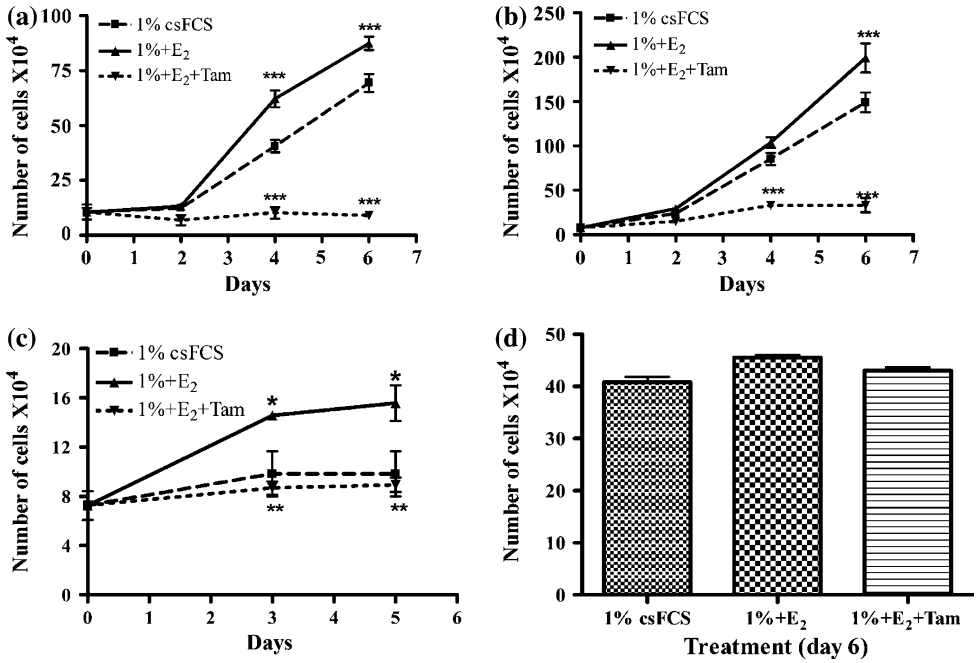

Fig. 3 Biological response of the LM05 cell lines to estradiol and tamoxifen. (**a**) LM05-E cells were seeded in 35 mm plates and treated for six days with vehicle, estradiol 10^{-8} M (E_2) or estradiol 10^{-8} M+ 4-OH-tamoxifen 10^{-6} M (Tam) in the presence of 1% charcoal stripped fetal calf serum (csFCS). As from day four estradiol significantly stimulated cell growth in comparison to the controls only treated with 1% csFCS (*** $P \leq 0.001$). Tamoxifen inhibited cell growth in comparison to both the estradiol treated groups and to the controls (*** $P \leq 0.001$). (**b**, **c**) The same experimental setting was carried out on LM05-Mix cells. In (**b**) an experiment where tamoxifen showed a high inhibitory activity is shown (*** $P \leq 0.001$). However, when cells organized and contact was established before the beginning of the treatment, the inhibitory effect of tamoxifen was greatly reduced as shown in (**c**). (**d**) LM05-F cells were treated with estradiol and 4-OH-tamoxifen as explained in (**a**). However, after 6 days of treatment estradiol did not stimulate or tamoxifen inhibit cell proliferation in a statistically significant manner. In all cases one of at least three experiments is shown

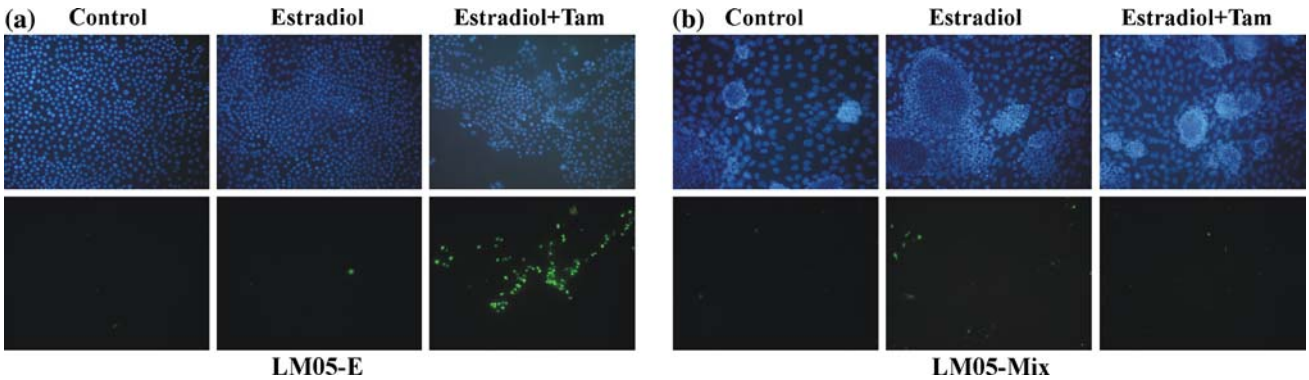

Fig. 4 Differential effect of tamoxifen on cell death in LM05-E and LM05-Mix cell lines. Forty thousand cells were plated on Labtek chamber slides and allowed to grow for 2 or 3 days until cell–cell contacts were established. At that time, cells were washed and treated for 48 h in phenol-red free media supplemented with 1%csFCS, with estradiol 10^{-8} M or estradiol+ 4-OH-tamoxifen 10^{-6} M. The Tunel assay was used to detect cell death (FITC, bottom row) and cells were counterstained with DAPI (blue, top row). As shown in the figure cell death was only induced in the LM05-E cells treated with tamoxifen, but not in the LM05-Mix cell line. Similar results were obtained when cell death was measured by propidium iodide staining (not shown)

The G-banding analysis of the metaphases of both cell lines revealed the presence of structural chromosome alterations. We observed that the LMO5-E cells presented two marker chromosomes as the result of translocations. The fluorescence in situ hybridization (FISH) technique using painting probes showed that the terminal region of one marker derived from chromosome 8 (Fig. 6a), and the proximal part of the other marker derived from chromosome 15 (Fig. 6b). In addition an isochromosome of chromosome 19 was found in some metaphases of LMO5-E. On the other hand LMO5-F cells presented two unidentified metacentric chromosomes, and a not yet

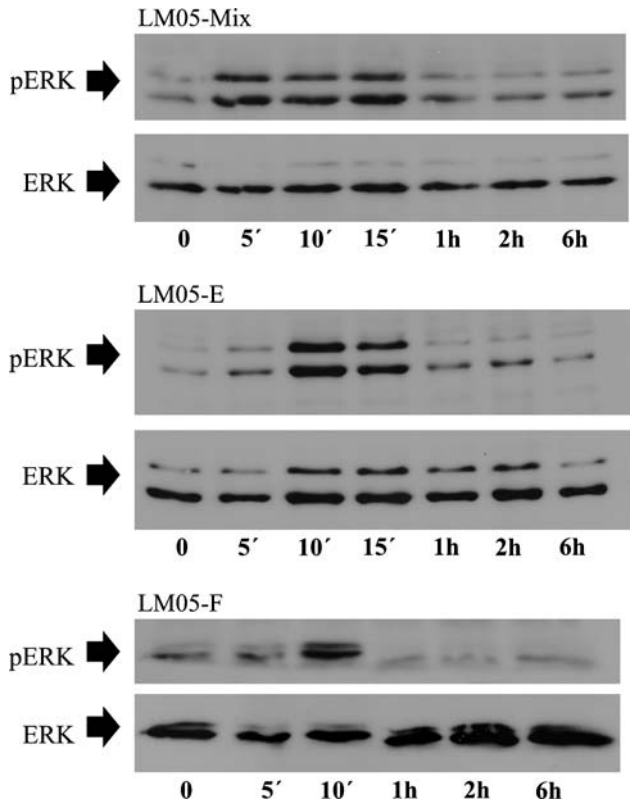

Fig. 5 Non-genomic action of estradiol on the LM05 cells. To investigate whether there were functional membrane estrogen receptors on the LM05-Mix, -E and -F cell lines, cells from the three cell lines were plated and allowed to grow to 60% confluency. At that time, they were washed and starved for 48 h in phenol-red free medium, time after which they were treated with estradiol 10^{-8} M for 0, 5, 10, 15 min and 1, 2 and 6 h. Phosphorylation of ERK1/2 was observed in the three cell lines between 5 and 10 min of treatment. After 1 h the phosphorylation levels were back to those observed at time cero. Controls were carried out with the corresponding dilution of absolute ethanol and no activation of ERK1/2 was observed (not shown). One of at least three experiments is shown for each cell line

identified Robertsonian translocation (Fig. 6c). Both cell lines also presented small unidentified acrocentric chromosomes. Numerical alterations involved chromosome 19 in gain in both cell lines. Some metaphases of the LMO5-E cells also presented losses of chromosomes 1 and 4, whereas the LMO5-F cell line showed gain in chromosome 15 and loses of chromosomes 12–14 and X. All together these results would suggest that the LM05-E and LM05-F cells are probably not related through a process of epithelial to mesenchymal transition, but most likely represent independent populations within the M05 tumor.

Discussion

In this paper we describe the characterization of three cell lines derived from the spontaneous estrogen dependent M05 mouse mammary tumor [13]. The original cell line established from the tumor, LM05-Mix, composed of an epithelial and a fibroblastic cell population, gave rise to the epithelial LM05-E and the fibroblastic LM05-F cell lines. We show here that although both cell populations expressed estrogen and progesterone receptors, their response to estradiol and tamoxifen with respect to proliferation and cell death was different. On the other hand the presence of membrane ER was suggested by non-genomic responses to estradiol. Finally we present the cytogenetic characterization of both the epithelial and the fibroblastic cells which suggests that these two cell types were generated from different populations within the M05 tumor, and not as a result of epithelial-mesenchymal transition amongst themselves.

Immunofluorescent analysis of the LM05-Mix cell line revealed that the epithelial cells were cytokeratin, E-cadherin and vimentin positive. On the other hand the fibroblastic cells were only positive for smooth muscle actin and vimentin. Interestingly, the LM05-Mix cell line proved to be very stable in its morphology and in the ratio of epithelial to mesenchymal cells. This cycle was maintained with every tripsinization, for over 40 passages (our unpublished observations). Traditionally primary cultures to establish cell lines from tumors have been carried out in such a way as to eliminate the fibroblastic compartment of the tumor. However, we know today that the stromal cells play a key role in tumor development and progression [5, 15]. Our LM05-Mix cell line shows that it is possible to establish cell lines that contain more than one cell type and that they are actually capable of growing stably together. This is important when considering that tumors are heterogeneous and that their behavior is the result of the interactions between the different cell types that compose them. The cytogenetic studies carried out on the LM05-E and LM05-F cell lines show that both populations present alterations. However, we did not find common markers between both cell lines suggesting that the LM05-F fibroblastic cells are not derived from the epithelial cells that were selected when the cell line was established. However, we cannot discard the possibility that they originated from an epithelial subpopulation that did not survive the culture conditions, or alternatively represent stromal fibroblasts that have undergone genetic modifications. The fact that the LM05-F cell showed genetic alterations is not surprising given that other authors have shown that both in human tumor samples and in mouse models fibroblasts may have an altered genome [4, 16]. Interestingly we found that the LM05-F cell line was tumorigenic when injected into syngeneic mice giving rise to undifferentiated tumors. On the other hand the LM05-E cell line proved to have a very low tumorigenicity as only in a few occasions did it generate tumors when more than one million cells were

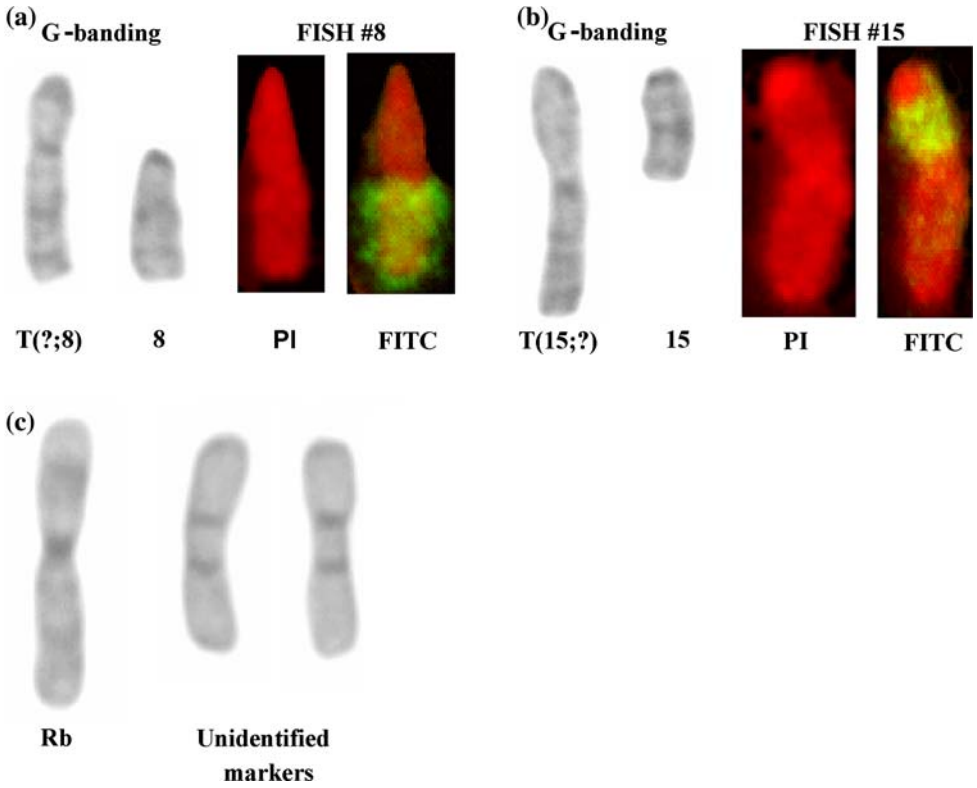

Fig. 6 Cytogenetic characterization of the LM05-E and LM05-F cell lines. Marker chromosomes of the cell lines LMO5 E (**a**, **b**) and LMO5 F (**c**), with banding and FISH techniques. (**a**) Translocation (T) between an unidentified chromosome and chromosome 8, T(?;8); and translocation between chromosome 15 and an unknown chromosome, T(15;?), observed by G-banding and FISH with a specific painting probe (detected with FITC-Avidin, in green). Chromosomes were counterstained with Propidium Iodide (PI). (**b**) Robertsonian translocation (Rb) and unidentified marker chromosomes, observed by G-banding

inoculated. Finally the LM05-Mix cell line gave rise to tumors that showed areas of differentiation as in the case of the parental tumor (our unpublished observations). Thus we believe that the LM05-Mix cell line is composed of two distinct tumor cell populations that are present in the M05 parental tumor, one of which is fibroblastic and the other epithelial. The generation of mixed models such as this one may be more physiologically relevant than single-cell cell lines to dissect in culture mechanisms involved in tumor biology.

From the LM05-Mix cell line the epithelial and fibroblastic cell populations could be separated employing a cloning procedure, giving rise to two new cell lines, LM05-E and LM05-F, respectively. Moreover, both cell lines stably maintained their unique characteristics for more than 40 passages as the parental LM05-Mix did (our unpublished observations). Both cell types (either cultured together or separately) proved to be positive for estrogen and progesterone receptors. As in the case of the M05 tumor from which the lines were generated [13], the LM05-E cell line was stimulated by estradiol and inhibited by tamoxifen. On the contrary, we found that at the level of cell proliferation the LM05-F cells did not respond to either treatment, although non genomic activation of the MAPK/ERK pathway was observed, as in the case of the LM05-E cells, with short pulses of estradiol. The biological role of this non-genomic signaling remains to be determined in our cell lines. Most interestingly, when testing the LM05-Mix cell line, we found that the sensitivity to tamoxifen was determined by the degree of cell–cell interaction between the fibroblastic and the epithelial cells, but not by interaction between the epithelial cells themselves, as proved by the cell death observed in 80% confluent LM05-E cultures. To date there are to our knowledge no studies analyzing the role of interactions between stromal and epithelial cells derived from the same tumor on resistance to tamoxifen. Recently, Shekhar et al. [12] showed the differential effect of fibroblasts derived from ER+/PR+ or ER−/PR− human tumors on the morphogenesis and response to tamoxifen of premalignant tamoxifen sensitive human breast cells, suggesting, like us, that the stroma may modulate endocrine resistance.

The most commonly used models to study the biology of estrogen responsiveness and tamoxifen resistance are the human MCF-7 [14] and ZR-75-1 [3] cell lines. Regarding mouse models, cell lines have been established from the estrogen dependent MXT mouse breast cancer model, which was induced by urethane [1, 17]. However, there are to our knowledge no cell lines derived from spontaneous estrogen dependent mouse mammary tumors. On the other hand the previously characterized cell lines are limited in the fact that they are constituted by only one cell type, which is the epithelial estrogen receptor positive cell. This has probably been one of the reasons why, in this field, theories to explain the mechanisms of tamoxifen resistance are mostly based on explanations that only consider

autocrine or paracrine loops between identical cells as responsible for progression. Thus, the LM05 cell lines, that count with both stromal and epithelial cells, present themselves as a new alternative and interesting model to understand in culture the biology of tamoxifen resistance in a context where paracrine interactions between different cell populations are present.

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