

## STROMAL-EPITHELIAL INTERACTIONS AND TAMOXIFEN RESISTANCE

**OSVALDO PONTIGGIA, GABRIEL FISZMAN, VANINA RODRIGUEZ,  
 ELISA BAL de KIER JOFFE, MARINA SIMIAN**

*Departamento de Cáncer Experimental, Instituto de Oncología Angel H. Roffo,  
 Facultad de Medicina, Universidad de Buenos Aires*

**Abstract** Tamoxifen, a selective estrogen receptor modulator, is the standard endocrine treatment for hormone receptor positive breast cancer, both in the initial adjuvant therapy and as treatment of patients with metastatic disease. However, about one third of patients with estrogen receptor (ER)- $\alpha$  positive tumors are refractory to tamoxifen therapy and a high percentage of patients who initially respond to tamoxifen develop resistance. Most breast cancers that acquire endocrine resistance retain ER- $\alpha$  expression, suggesting that loss of ER is not a common mechanism of resistance to endocrine therapy. Diverse signal transduction pathways influence the functional activity of ER, in addition to steroid ligand, and are critical in the responsiveness of tumors to anti-hormonal drugs. In particular, it is now well established that activation of several growth factor signaling cascades can promote resistance. However, although these pathways can be modulated by the tumor microenvironment, no studies to our knowledge have investigated this subject in an ER- $\alpha$  positive cell context. In this article we discuss the development of a new mouse model of estrogen responsive/tamoxifen sensitive breast cancer and propose that microenvironmental factors may be critical in the response to endocrine therapy, and could in the future be a rational target for the treatment of tamoxifen resistant breast cancer.

**Key words:** mammary cancer, tamoxifen

**Resumen** *Interacciones estroma-epitelial y resistencia al tamoxifeno.* El tamoxifeno, un modulador selectivo del receptor de estrógenos (RE), es el tratamiento de elección para pacientes con cáncer de mama RE<sup>+</sup>, tanto en terapia adyuvante, como para pacientes con metástasis. Sin embargo, alrededor de un tercio de los pacientes con tumores RE<sup>+</sup> son refractarios al tamoxifeno, y un alto porcentaje de pacientes que inicialmente responden a la terapia desarrollan resistencia. La mayoría de los tumores que adquieren resistencia al tamoxifeno mantienen la expresión de RE- $\alpha$ , sugiriendo que la pérdida del receptor no es un mecanismo común de resistencia. Varias vías de señalización modulan la actividad del RE y son críticas en la respuesta de tumores al tratamiento anti-hormonal. En particular, la activación de receptores para factores de crecimiento polipeptídicos han demostrado promover la resistencia. Sin embargo, a pesar de que estas mismas vías pueden ser moduladas por el microambiente tumoral, no hay hasta el momento trabajos que investiguen cuál sería su papel en un contexto RE<sup>+</sup>. En este artículo presentamos un nuevo modelo murino de cáncer de mama estrógeno dependiente y sensible al tamoxifeno, y proponemos que factores del microambiente tumoral podrían ser determinantes en la respuesta a la terapia hormonal.

**Palabras clave:** cáncer de mama, tamoxifeno

### Estrogen dependent breast cancer and available tumor models

Breast cancer is the most frequent cancer in women in industrialized countries (22% of all cancers) and is the second leading cause of cancer death<sup>1</sup>. 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. 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. However, 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, T-47D and ZR-75-1 have been the most widely used. They all express estrogen and progesterone receptors (PR) 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 among different cell types within the tumor. On the other hand *in vivo* experiments with these human cell lines are always restricted to immunosuppressed mice. As to syngeneic mouse models, only two have been used to understand the biology of hormone-dependent breast cancer: the MXT model, which was induced by urethane<sup>2</sup>, and the medroxyprogesterone-acetate induced mouse mammary tumors which express ER and PR but that are progesterone dependent<sup>3</sup>. There are to date, no publications of cell lines derived from spontaneous estrogen dependent mouse mammary tumors.

### The M05 tumor model

In our animal facility at the Instituto de Oncología Angel H. Roffo a spontaneous mouse mammary tumor arose in a one year old virgin female BALB/c mouse that was named M05. M05 has since been kept by successive subcutaneous syngeneic passages in female BALB/c mice. *In vivo* experiments determined that M05 is estrogen dependent for growth within the first eight passages, as it did not grow in ovariectomized mice, in male mice or in female mice treated with tamoxifen (Fig. 1). Only estradiol treatment of ovariectomized female mice restored tumor growth<sup>4</sup>. Immunofluorescence studies of the tumor tissue revealed the presence of both ER and PR. Histological analysis determined that M05 is a semi-differentiated adenocarcinoma with papillar differentiation. Confocal immunofluorescence studies of frozen sections showed that M05 is composed mostly of cytokeratin and E-cadherin positive epithelial cells that are organized in

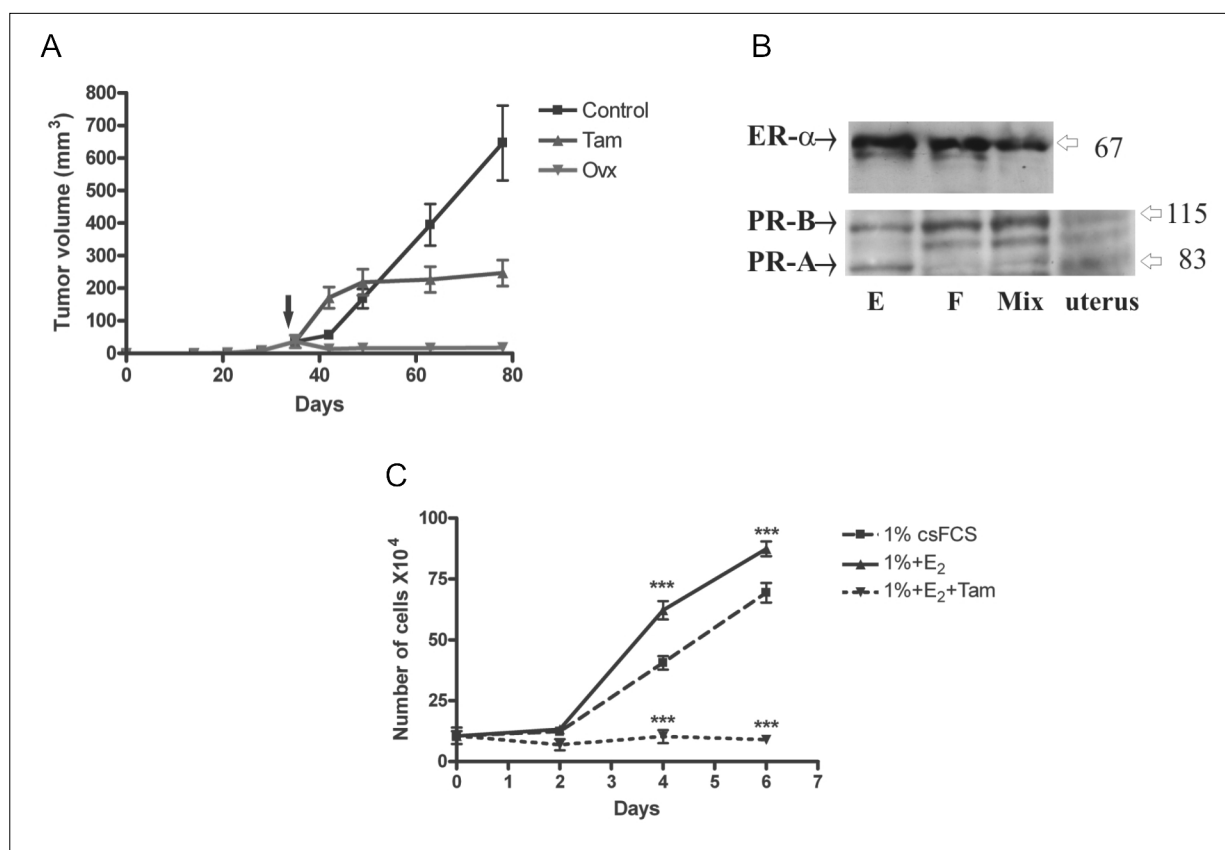


Fig. 1.– A) Growth curve of an early passage of the M05 tumor. The M05 tumor was inoculated s.c. in female BALB/c mice. At day 37 (arrow), once the tumors had reached an approximate size of 50 mm<sup>3</sup>, mice were ovariectomized, treated with tamoxifen (5 mg silastic pellets), or sham treated. Tumor growth was inhibited by both the ovariectomy treatment and by tamoxifen. B) Western blot showing the expression of ER-α and PR in the LM05 cell lines. A protein extract of mouse uterus was used as a positive control for PR. C) Proliferation assay showing that estradiol (E<sub>2</sub>) 10<sup>-8</sup>M stimulated cell proliferation of the LM05-E cells cultured in 1% charcoal stripped fetal calf serum (csFCS), whereas tamoxifen (10<sup>-6</sup>M) had an inhibitory effect. ANOVA, \*\*\*: Pd ≤ 0.001.

islands surrounded by vimentin positive, cytokeratin and E-cadherin negative stromal cells. Extracellular matrix components such as laminin, collagen IV and fibronectin are found mostly in the stromal compartment surrounding the epithelial cells (Fig. 1B). Thus, M05 is to our knowledge the first clinically relevant spontaneous estrogen dependent mouse mammary tumor model available for experimentation.

### The LM05 cell lines: a new model system to study stromal-epithelial interactions in the context of hormone-resistance

A primary culture obtained from a passage six M05 tumor gave rise to a continuous cell line named LM05-Mix. LM05-Mix is composed of two cell types, an epithelial and a fibroblastic cell population, that were subsequently cloned to generate the LM05-E and LM05-F continuous cell lines respectively. Immunofluorescence studies showed that the epithelial cell population was positive for E-cadherin, cytokeratins and vimentin, whereas the stromal cells were negative for cytokeratins and E-cadherin and positive for smooth muscle actin and vimentin. Analysis of extracellular matrix components showed deposition of laminin, collagen IV and

fibronectin as observed in the parental tumor. Staining was especially strong in areas of stromal-epithelial interactions. Finally, both cell types expressed ER and PR as determined by immunofluorescence and western blot. Thus, the LM05-Mix cell line turned out to recapitulate in culture the organization and characteristics of the M05 tumor.

Next, we were interested in investigating the response of the three cell lines to estradiol and tamoxifen. Proliferation studies were carried out in phenol red free medium supplemented with 1% charcoal stripped FCS. Fig. 1C shows that in the LM05-E cell line estradiol ( $10^{-8}$ M) caused a statistically significant stimulation of cell proliferation, whereas tamoxifen ( $10^{-6}$ M) was inhibitory. Similar results were obtained with the LM05-Mix cell line (not shown); however LM05-F did not reproducibly respond to either estradiol or tamoxifen in the same culture conditions. However, what drew our attention when working with the LM05-Mix cells was the fact that the inhibitory effect of tamoxifen was dependent on the degree of confluency of the cells at the beginning of the experiment, to the point that when cell-cell interactions were established before the treatment was introduced, tamoxifen did not seem to significantly affect cell proliferation. These results suggested that interactions between the two cell types could mediate tamoxifen resistance.

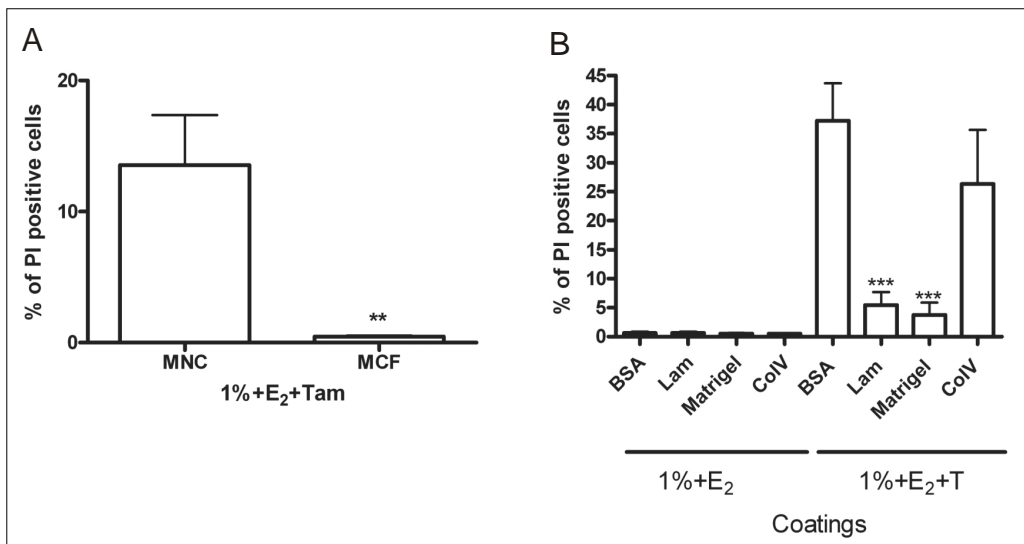


Fig. 2.— A) Protective effect of conditioned media derived from LM05-F cells. LM05-E cells were treated with 1%csFCS+ E<sub>2</sub> ( $10^{-8}$ M)+ tamoxifen ( $10^{-6}$ M) in the presence of LM05-F conditioned media (MCF), or in non-conditioned media (NCM) as a control. Apoptotic and necrotic cells were detected by propidium iodide (PI) staining of unfixed cells followed by DAPI counterstaining. The percentage of PI positive cells was calculated as an indicator of cell death (\*\*: Pd  $\leq$  0.01). B) Extracellular matrix components confer tamoxifen resistance. LM05-E cells were plated on laminin, matrigel or collagen IV. BSA was used as a control. They were then treated for 48 hrs with 1% csFCS+estradiol in the presence or absence of tamoxifen. Both laminin and matrigel significantly reduced the percentage of dead cells compared to the BSA control (\*\*\*: Pd  $\leq$  0.001).

## Stromal-epithelial interactions and tamoxifen resistance

To confirm the hypothesis that in the LM05-Mix cell line cell-cell interactions were mediating tamoxifen resistance, we cultured LM05-E and -Mix cell lines to 80% confluency and then treated the cells for 48 hrs with estradiol ( $10^{-8}$ M) in the presence and absence of tamoxifen ( $10^{-6}$ M). Apoptosis was detected by the TUNEL assay only. The LM05-E cells died in the presence of tamoxifen, whereas levels similar to the untreated group were observed in the LM05-Mix cells. Next, we set out to investigate what types of stromal factors could be modulating resistance to tamoxifen. Given the nature of cell-cell interactions we postulated that protection could be conferred through three basic mechanisms: a) through soluble factors, such as polypeptide growth factors, which have been associated to both normal and malignant stroma of the breast; b) through extracellular matrix components, which do not only act as a mechanical scaffold, but are able to initiate intracellular signaling cascades through their direct interaction with integrins or through release of bioactive fragments or c) via direct physical interactions between both cell types. As shown in Fig. 2A we found that soluble factors were able to confer resistance to tamoxifen. Treatment of LM05-E cells with tamoxifen in the presence of conditioned media prepared from LM05-F cells reduced cell death compared to non-conditioned media. Next, to investigate whether interaction with extracellular matrix components such as laminin and collagen IV were able to protect the epithelial cells, LM05-E cells were plated on purified laminin, collagen IV, matrigel or on BSA as a control. Fig. 2B shows that both laminin and matrigel conferred resistance, whereas collagen IV did not reduce the percentage of dead cells, compared to the BSA treated control. Thus our results suggest that microenvironmental factors, both soluble and present in the extracellular matrix are able to confer tamoxifen resistance.

## Discussion

In this article we have presented the development and characterization of a new murine syngeneic model of ER- $\alpha$  positive breast cancer. M05 is to our knowledge the first reported spontaneous mouse mammary tumor that is estrogen dependent and responds to tamoxifen *in vivo*. The LM05-Mix, -E and -F cell lines derived from it also express ER and PR and represent an invaluable model system to study not only how the epithelial compartment of the tumor responds to estradiol and tamoxifen, but also how cell-cell interactions modulate response to endocrine therapy. The results shown above suggest that microenvironmental cues may regulate tamoxifen resistance. Resistance to endocrine therapy is a major problem in

the management of ER- $\alpha$  positive breast cancer. In particular, activation of growth factor signaling pathways has been proposed in both *de novo* and acquired resistance to tamoxifen.

The main mechanism of *de novo* resistance of breast cancer cells to endocrine therapy is the loss of expression of ER. Evidence suggests that activation of growth factor signaling pathways might reduce ER- $\alpha$  expression and/or function. In particular, treatment of MCF-7 cells with EGF, IGF-1, TGF- $\beta$  or phorbol myristate acetate (TPA) reduced the levels of ER- $\alpha$  mRNA and protein<sup>5-8</sup>. Increased signaling through EGFR, PI3K/AKT, PKA and PKC were involved in this phenomenon. Moreover, heregulin, which activates both EGFR and ErbB-2, has been shown to suppress ER- $\alpha$  or its transcriptional activity<sup>9</sup>. Likewise, constitutively active ErbB-2, Raf1 or MEK resulted in a significant reduction in the expression of ER- $\alpha$  mRNA and protein, accompanied by a suppression on its transcriptional activity and the development of an estrogen independent phenotype<sup>10-13</sup>.

The EGFR/ErbB-2 signaling pathway has also been shown to be involved in acquired resistance to tamoxifen<sup>14</sup>. Nicholson's group established an *in vitro* tamoxifen resistant model derived from MCF-7 cells. These cells showed increased levels of expression of EGFR and ErbB-2, increased activation of EGFR/ErbB-2 heterodimers and increased phosphorylation of MAPK, AKT and nuclear ER in serine residues 118 and 167. Interestingly, the phosphorylation of ER and ER-induced transcription were increased by exogenous EGF-like peptides and was blocked by treatment with gefitinib. Signaling through IGF1-R has also been shown to be involved in tamoxifen resistance<sup>15</sup>, as has KGF<sup>16</sup>. Thus, growth factor signaling seems to be a key mechanism in the acquisition of resistance to tamoxifen.

As mentioned in the introduction to this article, most studies related to understanding the mechanisms leading to tamoxifen resistance have been carried out in MCF-7 cells. The examples given above illustrate the current hypothesis that explain both *de novo* and acquired resistance to tamoxifen. However, it seems surprising that the literature very seldom proposes the microenvironment as a player in the biology of ER- $\alpha$  positive breast cancer. In the last decade the tumor microenvironment has gained importance and today we accept that cancer is not only the result of a misregulation in oncogenes and tumor suppressors genes, but consider the tumor microenvironment as co-evolving with the mutated epithelium<sup>17</sup>. As an example Barcellos-Hoff has shown that modifications in the mammary gland stroma by ionizing radiation can promote the development of epithelial tumors from non malignant mammary epithelial cells<sup>18</sup>.

We show here that the LM05-E cells, that are tamoxifen-sensitive on their own, acquire a resistant behavior when their microenvironment is manipulated. Soluble

factors produced by the LM05-F cell line, together with purified growth factors (as previously shown by others) and extracellular matrix components modulate the response to tamoxifen. Although novel, these results are not surprising given the fact that integrins have been previously shown to activate the same signaling pathways as polypeptide growth factors, and actually cross-talk with various tyrosine kinase receptors. Understanding the mechanisms underlying our observations may open a new field of study in endocrine responsive breast cancer.

Great effort has been put in the last years to understand how breast cancer becomes refractory to endocrine treatments. However, little has been done to integrate the tumor microenvironment in these studies. Although treatments combining endocrine therapy with targeted intracellular small molecule inhibitors are currently in clinical trials<sup>19</sup>, very little basic research has been carried out from a tissue/organ perspective, where stromal and inflammatory cells together with the extracellular matrix are taken into account. As a result most of the current explanations are based on autocrine loops and alterations in the cancer cell itself. Confronting this problem from a more integrated perspective may prove to be a successful strategy in better understanding the progression of this disease and may lead to the development of novel therapeutic strategies.

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