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PRECLINICAL STUDY

A spontaneous estrogen dependent, tamoxifen sensitive mouse mammary tumor: a new model system to study hormone-responsiveness in immune competent mice

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Abstract Currently, an in vivo spontaneous model of estrogen dependent, tamoxifen sensitive breast cancer does not exist. We show here the characterization of the M05 mammary tumor that appeared spontaneously in a 1-year-old virgin female BALB/c mouse in our animal facility. The M05 tumor is a semi-differentiated adenocarcinoma that expresses estrogen and progesterone receptors. When it was transplanted to either male or ovariectomized female mice it did not grow. Moreover, ovariectomy or treatment with tamoxifen of tumor bearing mice led to a halt in tumor growth. Treatment of ovariectomized mice that had been inoculated with the M05 tumor showed that only estradiol, but not progesterone, promoted the re-growth of the tumor. Finally, after passage nine, tumor growth was achieved in male and ovariectomized female mice suggesting that the tumor had progressed to hormone independence. However, like often found in the clinic, expression of estrogen and progesterone receptors was maintained. This model mimics the biology of estrogen receptor positive breast cancer in humans and presents itself as an invaluable tool for the study of endocrine resistance in a physiologically relevant setting.

Keywords Breast cancer · Estrogen dependent · Tamoxifen sensitive · In vivo mouse model

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Introduction

Breast cancer represents the leading cause of cancer death among women in developed countries [11]. The concept that hormones are key contributors to carcinogenesis in mammary tumors emerged as early as 1896, when Beatson observed that elimination of ovarian function by oephorectomy could benefit women with breast cancer [2]. Today we know that approximately 70% of breast cancer patients are positive for estrogen and progesterone receptors, and approximately 50–60% of them benefit from endocrine therapy, being tamoxifen the mainstay treatment.

Estrogens are critical regulators of breast epithelial cell proliferation. ER-α is expressed in 15–30% of luminal epithelial cells present in normal breast tissue [18]. However estrogen dependent proliferation of breast epithelial cells is thought to occur in a paracrine manner, such that ER-α positive cells induce proliferation of ER-α negative cells via the secretion of growth factors [3]. In breast cancer, the action of estrogen is often deregulated and the percentage of cells expressing ER-α is dramatically increased together with a shift towards an autocrine response, given that in malignant tissue estrogen directly stimulates growth [17].

Evidences implicating estrogen as a key breast carcinogen come from various lines of investigation. In rats a high incidence of mammary tumors can be induced by prolonged treatment with estrogens. In mice, however,

prolonged estrogen treatment rarely results in mammary tumors in the absence of the mouse mammary tumor virus [16]. A strong positive association between breast cancer risk and circulating levels of estrogens has now been well confirmed in humans; women with hormone levels in the top 20% of the distribution have a 2- to 3-fold higher risk of breast cancer versus the bottom 20% [5].

Most studies carried out to understand the biology of steroid receptors in the context of the progression of breast cancer have been done utilizing the MCF-7 breast cancer cell line [19], and to a lesser extent the T-47D [9] and the ZR-75-1 cell lines [6]. In vivo experiments in all three cases have been restricted to immune deficient mice, given the human origin of the cell lines. Regarding mouse models, the MXT tumor line is an estrogen dependent mouse mammary tumor that was induced by urethane [21]. On the other hand progesterone dependent tumors were generated by long term administration of medroxyprogesterone acetate [12]. However, to date there are no descriptions of spontaneous estrogen dependent mouse mammary tumors in mice.

In the present study, we describe a spontaneous hormone-dependent mouse mammary tumor (M05) that appeared in a virgin BALB/c mouse of our breeding colony. We show that it expresses ER and PR and that its growth is estrogen dependent and sensitive to ovariectomy and treatment with tamoxifen in early passages. As with human tumors, it becomes hormone independent with time, but does not lose the expression of steroid receptors. We believe it represents a new and valuable model to study the biology and progression of hormone dependent breast cancer.

Materials and methods

Animals

Inbred BALB/c female mice, 2–4 month old were obtained from our Animal Care Division. All animals were kept in metal cages (5–6 per cage) with controlled temperature (22 ± 1°C) and light exposure of 12 h. Food and water were available ad libitum. Animal care and manipulation were in agreement with institutional guidelines and the Guide for the Care and Use of Laboratory Animals [8].

Tumor and transplantation procedures

The original tumor appeared spontaneously in a mammary gland of a 12-month old virgin inbred female mouse of our BALB/c colony. The tumor was removed under aseptic conditions and the necrotic tissue was eliminated. It was minced into 1 mm^3 pieces and transplanted s.c. into the lateral flank of female mice with a trocar. After the 5th transplant, tumors were removed and frozen in liquid N_2. The M05 tumor is maintained in our laboratory by serial transplants in syngeneic mice performed every 7–8 weeks.

Tumors were measured twice a week with a Vernier caliper in two different planes (height and width). At autopsy tumors were weighed and kept in formalin, or frozen on dry ice and kept at −80°C.

Chemicals

Estradiol (β-Estradiol) and progesterone (6α-Methyl-17α-hydroxy-progesterone) were obtained from Sigma Chemical Co., St. Louis, MO. Tamoxifen citrate was kindly provided by Gador S.A, Buenos Aires Argentina.

Hormone dependency studies

For ovariectomy studies both ovaries (bilateral ovariectomy) of 8 week old female mice were removed via a small midline incision of the abdominal wall under general anesthesia by s.c. administration of 0.01 ml/g of body weight of a cocktail of Ketamine (Ketalar, Parke Davis 0.23 mg/ml) and Rompum (Bayer, 0.14 mg/ml). Mice were ovariectomized approximately 1 month before, or 1 month after the tumor transplant. To confirm the surgical castration vaginal smears were obtained twice a week at the same hour of the day. Estradiol solutions used for injection were prepared extemporaneously by appropriate dilution with sterile saline of a stock ethanolic solution (2.5 mg E_2/ml). Mice were treated twice a week by s.c. injection of 2.5 μg in a final volume of 0.1 ml. Progesterone was dissolved in sesame oil of stock progesterone-DMSO (25 mg Pg/ml). Mice were treated once a week by s.c. injection of 2.5 mg dissolved in 0.1 ml. The solutions were maintained at 4°C. Finally, tamoxifen was administered as 5 mg silastic pellets. Vehicle or empty pellets were used as controls.

Histology and immunofluorescence

Specimens were fixed in 10% formalin, dehydrated and embedded in paraffin. Sections of approximately 3 μm of thickness were stained with hematoxylin and eosin and examined under a microscope. For immunofluorescence studies frozen tissues were mounted in OCT and 15–20 μm thick sections were cut using a cryostat. They were air dried and fixed for 20 min in 10% formalin in PBS. Subsequently, they were permeabilized by incubating in 0.1% Triton X-100 in PBS for 30 min at 37°C, and after that non-specific binding sites were blocked by incubation in

blocking buffer (PBS containing 2% fetal calf serum) for 1 h at room temperature. Sections were then treated with primary antibodies dissolved in blocking buffer at a 1/100 dilution ON at 4°C. After three washes in PBS sections were incubated with the corresponding fluorescein conjugated secondary antibodies (1/100 dilution) for 1 h at room temperature in blocking buffer. Slides were then washed with PBS and nuclei were stained with propidium iodide and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Sections were analyzed under a Nikon Laser Confocal Microscope.

Antibodies

The following primary antibodies were used for immunofluorescence studies: rabbit anti-laminin (E.Y. Laboratories, San Mateo, CA) a kind gift of Dr Mina J. Bissell, rabbit anti-collagen IV (a kind gift of Dr Mina J. Bissell), rabbit anti-E-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-estrogen receptor-α (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-progesterone receptor (Santa Cruz Biotechnology, Ca), sheep anti-estrogen receptor-β (a kind gift of Dr. Gustaffson), goat anti-estrogen receptor-β (Santa Cruz Biotechnology, Ca), mouse anti-cytokeratin 8 (Abcam), mouse anti-cytokeratin 14 (Sigma). 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). In the case of the experiments using mouse primary antibodies, tissues were incubated with Mouse on Mouse reagent (M.O.M., Vector Laboratories) to block any endogenous mouse antibodies, as indicated by the manufacturer.

Statistical analysis

The significance of the differences in the assays were analyzed by Student's *t*-test or two way Anova.

Results

The M05 tumor is a mouse mammary adenocarcinoma that is positive for estrogen and progesterone receptors

The M05 mouse mammary tumor appeared spontaneously in the fourth mammary gland of a 12 month old female virgin mouse in our breeding colony. The histopathology revealed a semi-differentiated adenocarcinoma with a very polymorphic pattern that included acinar/glandular areas co-existing with solid sheets or cords of tumor cells, as well as areas of cribiform or papillary aspect (Fig. 1a). Tumor parenchyma was surrounded by a rather abundant vascularized stroma, constituted by large fibroblast-like cells and a predominantly amorphous extracellular matrix (Fig. 1b). Gland-like structures were surrounded by a luminal layer of cuboidal or cylindrical tumoral cells delimited by an outer layer of myoepithelial-like cells (Fig. 1b). Frequently glands were highly dilated or presented secretions in their lumens. Tumor cell nuclei were only slightly atypical and mitotic figures were frequently found. These morphological characteristics of the parental tumor were maintained throughout the serial in vivo passages. Immunofluorescence studies revealed that the parenchyma was mostly composed of cytokeratin-8/E-cadherin positive epithelial cells with cytokeratin-14 positive cells mostly found in a basal position, in concordance with their myoepithelial appearance (Fig. 2). Fibroblastic cells in the stroma stained for vimentin (Fig. 2). Extracellular matrix components

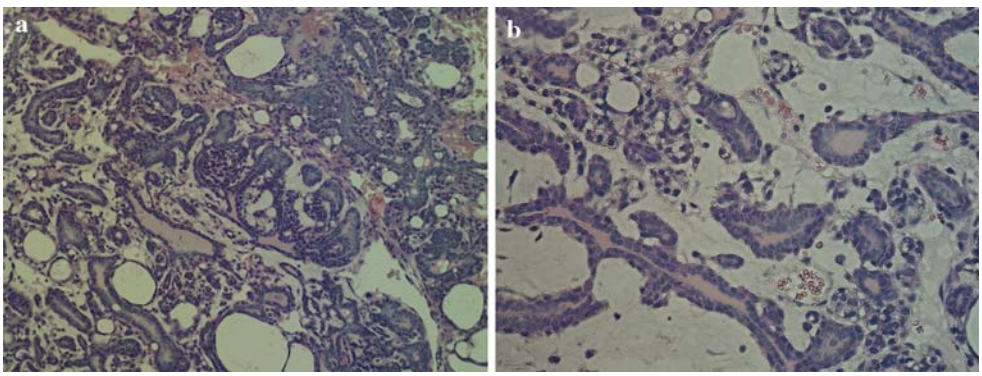

Fig. 1 Histology of the M05 primary tumor. (**a**) Hematoxylin and eosin stained section of the M05 tumor shows a semi-differentiated adenocarcinoma with a very polymorphic pattern with acinar/glandular areas co-existing with solid sheets or cords of tumor cells, as well as areas of cribiform or papillary aspect (200×). (**b**) Tumor parenchyma is surrounded by an abundant vascularized stroma, constituted by large fibroblast-like cells and a predominantly amorphous extracellular matrix. Gland-like structures are surrounded by a luminal layer of cuboidal or cylindrical tumoral cells delimited by an outer layer of myoepithelial-like cells (400×)

such as laminin (Fig. 2), fibronectin and collagen IV (not shown) appeared around the epithelial islands in a basement membrane like distribution. Staining for ER-α, β and PR revealed that 100% of the cells were positive for the three receptors, with some cells staining more intensely than others (Fig. 2), raising the question of whether the behavior of the tumor could be hormone responsive.

Growth of the M05 tumor in female, male and ovariectomized mice

When the M05 tumor was transplanted to syngeneic female and male mice, it only grew in female mice, suggesting that it could be a hormone dependent mammary tumor (Fig. 3). Moreover, tumor growth was increased in pregnant mice compared to virgin (not shown). To confirm this hypothesis, the tumor was transplanted to intact and ovariectomized female mice. As shown in Fig. 4a, the tumor progressed only in the intact female mice, but did not grow in the ovariectomized counterparts. Furthermore, to verify whether established tumors were affected by a decrease in hormone levels, tumors were transplanted s.c. and after reaching an average diameter of 12 mm, half of the animals were ovariectomized. Figure 4b depicts that tumor growth ceased in the treated group, as compared to the controls. We did not observe, however, tumor remission under these experimental conditions.

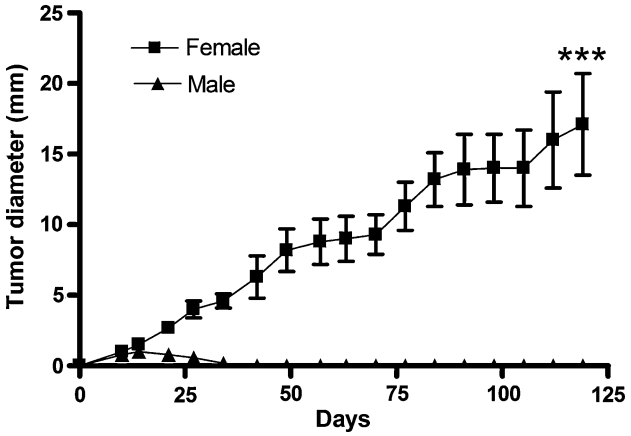

Fig. 3 Growth of the M05 tumor in female and male mice. The M05 tumor was inoculated in female ($n = 6$) and male ($n = 5$) mice. Tumor diameter was measured at least once a week. Only in female mice tumors developed, whereas in male mice no tumors were detected even 120 days after the inoculation ($P \leq 0.001$). One of at least three independent experiments is shown

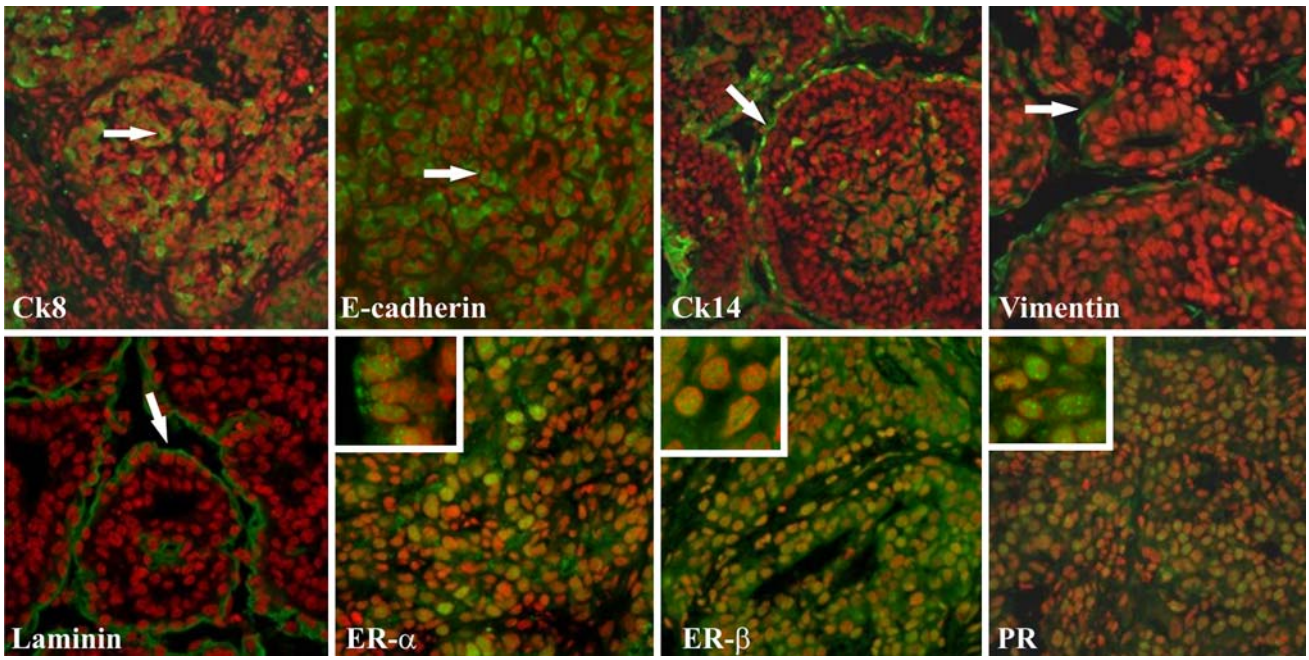

Fig. 2 Characterization of the M05 tumor by immunofluorescence. Frozen sections of the M05 tumor were stained for cytokeratin 8, E-cadherin, cytokeratin-14, vimentin, laminin, estrogen receptor-α, estrogen receptor-β and progesterone receptor. In all cases secondary antibodies were conjugated to FITC (green) and nuclei were counterstained with propidium iodide (red). Arrows indicate positive (green) cells for each of the analyzed cell markers. Insets in figures corresponding to estrogen and progesterone receptors show that both nuclear and cytoplasmatic stainings were detected. For all receptors 100% of the cells stained positive (sections from at least three independent tumors were analyzed), with some cells staining stronger than others. Magnification: 400 and 600×

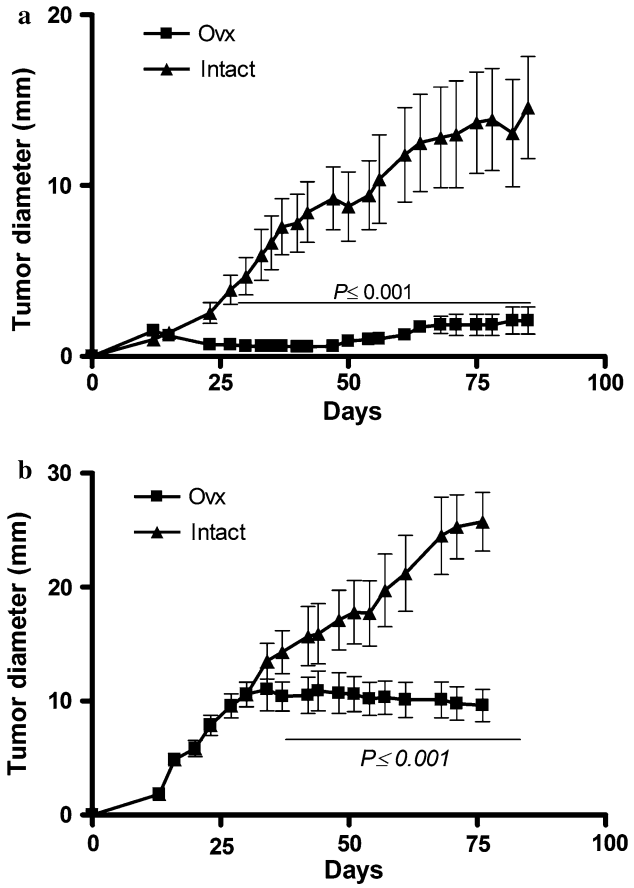

Fig. 4 Growth of the M05 tumor in intact and ovariectomized mice. (**a**) The M05 tumor was inoculated in intact and ovariectomized (Ovx) mice ($n = 10$ per group). Tumor diameter was measured twice a week. As from day 23, tumor diameter was significantly larger in the intact mice, compared to the Ovx group ($P \leq 0.001$). (**b**) To test whether ovariectomy affected the growth of established tumors, thirteen virgin female mice were inoculated with the M05 tumor and at day 30, when they had reached an average diameter of 10 mm, seven of the mice were ovariectomized (Ovx), and the remaining six were left intact. Ovariectomy significantly inhibited tumor growth as from day 37 ($P \leq 0.001$). One of at least three experiments is shown

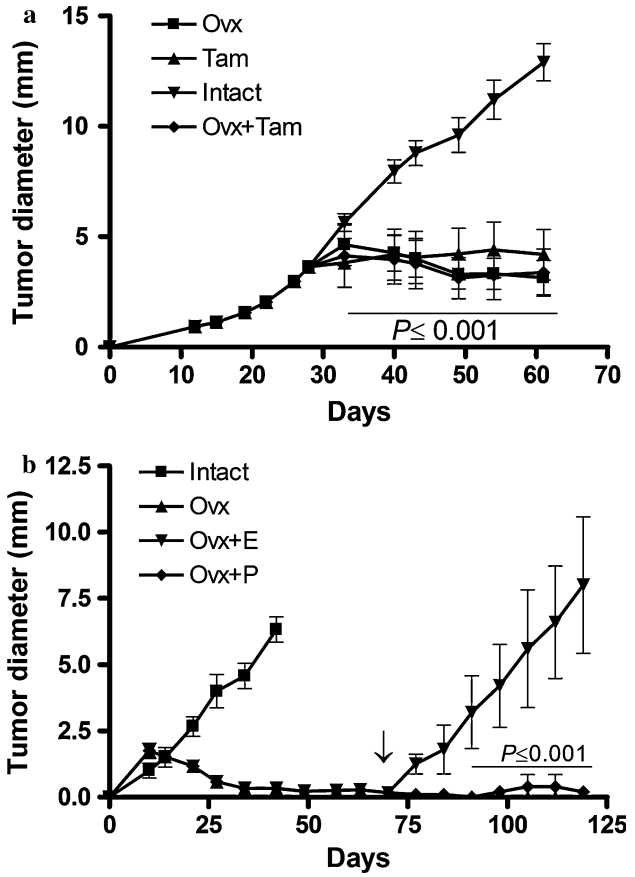

Fig. 5 Tamoxifen inhibits and estradiol promotes the growth of the M05 tumor. (**a**) Forty mice were inoculated with the M05 tumor. At day 28 when the tumors had reached an average diameter of 3,63 mm, they were randomly divided into 4 groups of $n = 10$ mice each and were either ovariectomized (Ovx), treated with tamoxifen (Tam), ovariectomized and treated with tamoxifen (Ovx + Tam) or left untreated. As from day 33 mice corresponding to any of the treated groups were significantly smaller than those in the untreated group ($P \leq 0.001$). (**b**) To test whether estrogen and/or progesterone promoted tumor growth, 6 intact and 14 ovariectomized mice were inoculated with the M05 tumor. At day seventy (arrow) the ovariectomized mice were further treated with estradiol (Ovx + E, $n = 4$), progesterone (Ovx + P, $n = 5$) or with vehicle (Ovx, $n = 5$). Only in the group treated with estradiol did tumor growth resume, with tumor diameters being significantly larger than the Ovx groups as from day 91 ($P \leq 0.001$). One of at least three experiments is shown

The M05 tumor is tamoxifen sensitive and estrogen dependent

Having observed that the growth of the M05 tumor was affected by ovariectomy we next tested whether tamoxifen could inhibit tumor growth. To do so we inoculated the M05 tumor to female virgin syngeneic mice and when it reached an average size of 5 mm we treated mice with either tamoxifen, ovariectomy or both treatments, and a group was left untreated. As observed with ovariectomy, treatment with tamoxifen inhibited tumor growth in a statistically significant manner, as compared to controls (Fig. 5a). The combination of both treatments did not prove to be additive, suggesting that the effect of the ovariectomy was mediated by the down regulation in the levels of estradiol.

Next, to confirm that the tumor was estrogen dependent, intact and ovariectomized mice were inoculated with the M05 tumor. At day 70, once lack of tumor growth was confirmed in the ovariectomized group, mice were further treated with estradiol, progesterone or with vehicle. Tumor growth only resumed in the group treated with estradiol (Fig. 5b), implicating again that M05 was an estrogen dependent mouse mammary tumor.

Progression of the M05 tumor to hormone independence

One of the hallmarks of ER-α positive breast cancer in humans is that it eventually progresses and becomes hormone independent. In the M05 tumor model a similar situation was found, given that after passage nine tumors began to grow in male mice (Fig. 6a). Moreover, as expected, this was also observed in ovariectomized mice (Fig. 6b) implying that at this stage the tumor no longer required estrogen to grow. Immunofluorescence staining confirmed the presence of ER-α, ER-β and PR suggesting that, like often found in the clinic, the loss of hormone-dependence was not due to a loss of expression of steroid receptors (Fig. 7).

Discussion

In this paper we describe the characterization of, to our knowledge, the first spontaneous mouse model of estrogen dependent breast cancer. We show here the morphological features, the expression of steroid receptors, as well as the response to hormonal treatments that allowed us to establish that the M05 tumor is estrogen dependent and tamoxifen sensitive. It represents a new and valuable tool to study hormone-responsiveness in an immunocompetent setting.

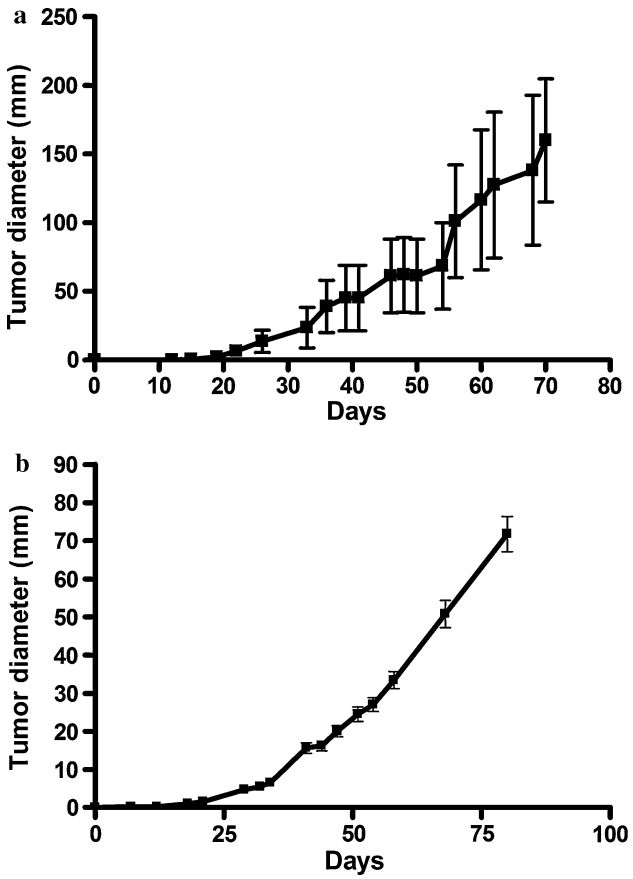

Fig. 6 Late passage M05 tumors grow in male and ovariectomized mice. Four male mice (Fig. 6a) and ten female ovariectomized mice (Fig. 6b) were inoculated with passage 15 M05 tumor. As shown in both graphs, and contrary to what was shown for early passage M05 tumors, growth was established and maintained in these conditions. One of a two experiments is shown

To date reports analyzing in vivo response to tamoxifen or other SERMs or even aromatase inhibitors are mostly carried out using MCF-7, T47-D or the ZR-75-1 breast cancer cell lines. One of the main pitfalls regarding these model systems is the fact that these cell lines are not derived from primary breast tumors, but from tumor metastasis, especially aspirates or pleural effusions.

Second, and not least important, is the fact that the use of human cell lines in vivo is restricted to immune compromised mice. This presents two mayor problems: one is the lack of an immune system that we know is essential in modulating tumor progression [4]. On the other hand tumors are not only composed of malignant epithelial cells, but stromal fibroblasts are key players in tumor progression. In the mammary gland in particular, fibroblasts can modulate the progression of otherwise non-malignant epithelial cells [1]. To address this issue attempts to reconstitute the human mammary stroma in immune deficient mice have been carried out [10]. However, probably due to technical issues, to date ER positive human cell lines have not been used in this experimental setting. Finally, there are a couple of transgenic models such as the p53 null [14], the *neu* [15] and the SV_{40} Large Tag model [22] where premalignant progression of the breast is hormone responsive as evidenced by delayed tumorigenesis in ovariectomized and tamoxifen treated mice. However none have been reported as adequate models for the study of the progression to tamoxifen resistance. Thus, the M05 tumor presents itself as a physiologically relevant model due to the fact that it allows the study of the progression of an estrogen responsive, tamoxifen sensitive tumor in an adequate microenvironment.

Morphological analysis of the M05 tumor revealed that it is a semidifferentiated adenocarcinoma with papillary areas. Immunofluorescence studies showed that the parenchyma is composed of both luminal cytokeratin-8 positive cells, and basal cytokeratin-14 positive cells. On the other hand stromal-like cells stained positive for vimentin. Staining for extracellular matrix components showed expression of laminin, collagen IV and fibronectin. Breast tumors with a papillary morphology have been produced by a variety of transgenes including the PyV-MT [7] and met-1 [13] transgenes. In humans these tumors are less frequent accounting for 2% of all breast malignancies [20]. Interestingly, although the M05 tumor morphologically resembles a less frequent type of human breast cancer, its

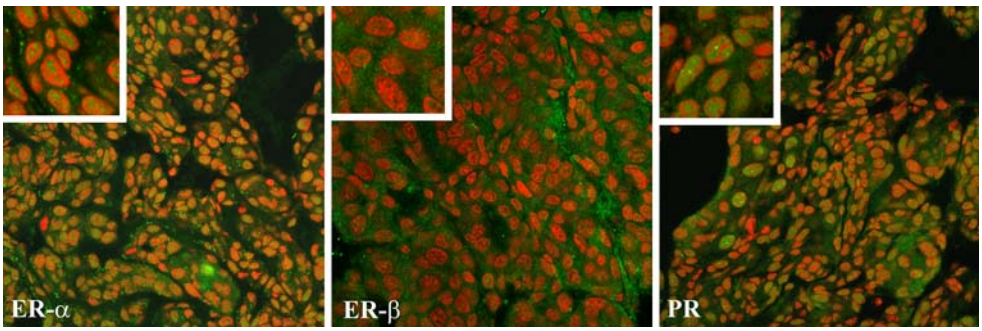

Fig. 7 Expression of ER-α, β and PR in late passage M05 tumors. Frozen sections of passage 15 M05 tumors were stained for estrogen receptor-α, estrogen receptor-β and progesterone receptor. In all cases secondary antibodies were conjugated to FITC (green) and nuclei were counterstained with propidium iodide (red). As shown in the insets both nuclear and cytoplasmatic stainings were detected. Analysis of at least three independent late passage tumors showed that 100% of the cells were positive for estrogen and progesterone receptors, with some cells staining stronger than others. Magnification: 600×

biological behavior is rare amongst mouse models given that, like 50% of human breast cancers, it is hormone sensitive. Mouse tumors, as mentioned above, are generally not hormone dependent [16].

One of the main problems in the clinic regarding the management of breast cancer is the progression to hormone-independence. In the M05 tumor we found that around passage nine tumors began to grow in ovariectomized and male mice, thus implying that they had become estrogen independent. However, the expression of both estrogen and progesterone receptors was maintained, suggesting that like in the human setting, changes in the signaling pathways involving estrogen receptors, but not the loss of expression, may be involved in hormone-independence.

We have characterized a new clinically relevant murine model of estrogen dependent, tamoxifen sensitive breast cancer. We believe the use of the M05 tumor will be an invaluable tool to understand how tumor-host interactions may influence the development of resistance to endocrine therapy, a yet unsolved problem in the clinic.

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