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Report

Prostate-specific antigen induction by a steroid hormone in T47D cells growing in SCID mice

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Summary

Previous studies revealed that prostate-specific antigen (PSA) is present in > 30% of human breast tumor cytosols. Survival analysis showed that patients with PSA-producing tumors have a reduced risk for relapse, suggesting PSA to be an independent favorable prognostic marker for a large subset of breast cancer patients. The present investigation established an *in vivo* model for the induction of PSA in human breast cancer tumors growing as xenografts in severe combined immunodeficient (SCID) mice. The human mammary cancer cell-line T47D was grown i.m. in female mice. When the tumor and leg diameter reached 10 mm, the mice were stimulated daily with norgestrel for either 5 or 7 days to produce PSA, and sacrificed on day 8. The prostate cancer cell-line LNCaP was grown in male mice and functioned as a positive control for PSA production. After T47D and LNCaP mice were sacrificed, a highly sensitive immunofluorometric assay was used to analyze the PSA concentration in the tumor, muscle, liver, and kidney cytosols. Norgestrel-stimulated T47D mice showed significantly more PSA in the tumors compared to tumors of the control mice. However, PSA levels in tumors of the stimulated mice were significantly lower than those in the LNCaP xenografts. No PSA levels above background were present in the blood and normal tissue of the norgestrel-stimulated or control T47D xenografts. This mouse model will be a valuable tool for investigating and screening new therapies for a subgroup of breast cancer patients who have significant PSA concentrations in their tumors.

Introduction

Prostate-specific antigen (PSA) is a *M*r 33,000 single chain glycoprotein [1]. The human gene encoding PSA was cloned and sequenced, and was localized to the long arm of chromosome 19 [2]. PSA is a kallikrein-like serine protease with a chymotrypsin-like activity. It is present in semen at concentrations of 0.5–3.0 g/liter, and participates in semen liquefaction after ejaculation [3]. Serum of normal men contains PSA levels below 4 μg/liter. However, there are higher PSA levels in the serum of patients with benign diseases of the prostate and prostatic carcinomas. The concentration of PSA in serum is a valuable biological marker for screening, diagnosis, and monitoring of patients with prostate cancer [1].

Until recently, it was believed that only epithelial cells of the prostate produced PSA. Recent studies, making use of ultrasensitive detection-techniques

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for PSA, have shown that other human tissues and tumors can produce PSA at low levels. For example, colon, ovarian, liver, kidney, and adrenal tumors can produce PSA [4–6]. PSA immunoreactivity was also examined in a cohort of more than twelve hundred breast cancer patients, with a sensitive time-resolved immunofluorometric assay [7, 8]. Results showed that immunoreactive PSA was present in greater than 30% of the breast tumor cytosols, at concentrations greater than 30 pg/mg of total protein. Studies confirmed that the immunoreactive PSA detected in the breast tumor cytosols, was identical to the PSA produced by the prostate gland [9].

Breast cancer patients with PSA-positive tumors are a subset among the patients with steroid-hormone receptor-positive tumors. Multivariate analysis showed that patients with PSA-producing tumors have a reduced risk for relapse. Thus, PSA is an independent favorable prognostic marker for a large subset of breast cancer patients [10].

Breast cancer is a heterogeneous disease. Breast cancer patients are usually divided into subgroups based on different prognostic and predictive markers. These markers are used to classify patients into high or low risk groups in terms of treatment decisions after local surgery [11]. Although a variety of markers are available, physicians cannot readily identify patients who may benefit from adjuvant treatment. The identification process has been difficult because the available markers are not specific enough [10]. To better understand the pathology of breast cancer and define the therapeutic options for the different subgroups, it is necessary to identify and characterize new prognostic markers such as PSA.

According to Young et al. [12], androgenic steroids bind to androgen receptors and regulate transcription of the PSA gene. However, steroid hormones and their receptors share extensive structural similarities. The progesterone, androgen, glucocorticoid, and mineralocorticoid receptors recognize highly homologous hormone response elements (HRE) on DNA, and could all regulate genes whose promoters contain such response elements [13]. Clinical data suggested PSA to be upregulated in some tumors and not others [8]. To investigate the mechanism of PSA gene regulation in the breast, Yu et al. [14] developed an *in vitro* system that reproduced this PSA production by breast tumor cells. They found that steroid hormones such as progestins can induce steroid-hormone receptor-positive breast cancer cell-lines to produce PSA *in vitro*. Furthermore, estrogens failed to induce such stimulation and could block PSA induction by androgens in T47D cells. Norgestrel was shown to be a strong PSA-inducing progestin.

The present investigation establishes an *in vivo* model for PSA gene induction as an extension of the *in vitro* model of Yu et al. [14]. The human breast cancer cell-line T47D and the prostate cancer cell-line LNCaP were grown in SCID mice, and the ability of norgestrel to induce PSA production in the breast tumors was investigated. Our results show that it is possible to induce PSA production *in vivo* using the T47D SCID mouse xenograft system.

Materials and methods

Cell lines

The steroid-hormone receptor-positive breast cancer cell-line T47D and the prostate cancer cell-line LNCaP were obtained from ATCC (Rockville, MD). Cells were grown initially in flasks at 37 °C and 5% CO_2 in Alpha medium supplemented with antibiotics and 10% fetal calf serum (growth medium). Typical cell doubling times were 20–30 hours. The cells were then detached by trypsin, counted with a particle counter and hemocytometer (to assess clumping), washed, and resuspended in growth medium for injection into animals.

Animals

Female and male SCID mice with a Balb/c genetic background (eight-ten weeks old) were obtained from the breeding colony of Ontario Cancer Institute (Toronto, Canada). Animals were kept in micro-isolator cages that were changed twice weekly. Mice received a supply of sterile water and gamma-irradiated rodent food (Teklad, WI; 5% rodent diet

7012) *ad libitum*. To prevent contamination, the animals were handled under a laminar flow hood.

Generation of SCID mice xenografts and PSA induction

In vivo growth was initiated by a single i.m. injection of cells into the left hind leg of mice. 3×10^6 T47D cells were injected into female mice (T47D mice), and 5×10^6 LNCaP cells were injected into male mice (LNCaP mice). All cells were injected in a volume of $\sim$ 0.05 ml growth medium. To stimulate tumor growth, T47D mice were injected s.c. in the scapular region with 0.1 ml of β-estradiol 17-valerate (5 mg/ml in sesame oil; Sigma Chemical Co., St. Louis, MO) once every two weeks, beginning 1 week after cell injection [15]. When tumors grew to a leg diameter (leg plus tumor) of 10 mm ($\sim$ 0.5 g of tumor), β-estradiol 17-valerate injections were stopped, and T47D mice were randomly divided into three groups. One group was stimulated daily with 0.05 ml of norgestrel (1 mg/ml in sesame oil; Sigma Chemical Co., St. Louis, MO) for five days. The second group was stimulated daily with 0.05 ml norgestrel (1 mg/ml in sesame oil) for seven days. The third group was stimulated daily with 0.05 ml of the vehicle (sesame oil; Sigma Chemical Co., St. Louis, MO) for seven days. In all stimulation experiments, mice were injected s.c. in the scapular region. All mice were sacrificed by cervical dislocation 8 days after the first norgestrel/vehicle injection. Mice injected with the LNCaP cell-line received no further treatment and were sacrificed when tumor and leg size reached 10 mm in diameter. The following tissues were obtained from each mouse: tumor, muscle (from the opposite leg), liver, and kidneys. All tissues were immediately frozen in liquid nitrogen and stored at – 70 °C until tissue extraction.

Preparation of tissue extracts and PSA assay

Three samples ($\sim$ 10 mg each) were obtained from different regions of each frozen tumor or normal tissue. Each tissue sample was pulverized to a fine powder at – 70 °C. The tissues were then lysed for 30 min on ice, using 0.5 ml of a lysis buffer [50 mM Tris buffer (pH = 8.0) containing 150 mmol of NaCl, 5 mmol of EDTA, 10 g of NP40 surfactant, and 1 mmol of phenylmethyl sulfonyl fluoride/liter]. Lysates were centrifuged at 15,000 g at 4 °C for 30 min, and the supernatants were collected for PSA and total protein analyses. Blood samples were collected in heparinized tubes, centrifuged, and the supernatants removed for PSA analysis. A time-resolved immunofluorometric assay for PSA was used and has been described previously by Ferguson et al. [16]. The detection limit of the assay is 1–2 ng PSA/liter. Using this assay, the PSA content of all tissue extracts was measured in duplicate and compared to a standard curve, which was run daily for each assay. Within-run imprecision of the assay is $\sim$ 6% and between-day imprecision is $\sim$ 5% [16].

PSA immunoreactivity

The relative amount of immunoreactive PSA in each tissue was calculated by dividing each PSA value by the total protein in each sample to yield pg PSA/mg protein (PSA level). Total protein in each sample was measured in duplicate using the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL).

Immunohistochemistry

LNCaP tumors were fixed in formaldehyde, sectioned and stained for PSA using an indirect labelling method that detects antigen sites in the specimen. First, tumor sections were incubated with a primary polyclonal rabbit anti-PSA antibody (DAKO Diagnostic Canada Inc., Mississauga, Ontario) which was diluted 1:800. Then, a biotinylated secondary antibody (Signet Laboratories, Inc., Dedham, MA) was diluted 1:5 and incubated with the samples. The sections were then incubated with a peroxidase-conjugated streptavidin-biotin complex (Signet Laboratories, Inc., Dedham, MA), followed by the addition of a solution containing the chromogenic substrate, 3,3'-diaminobenzidine te-

trahydrochloride (DAB; Sigma Chemical Co., St. Lous, MO), and hydrogen peroxide. This produced localized coloured precipitate at the sites of antigen. Haematoxylin was used as a counterstain to visualize all cells.

Statistical analysis

PSA levels in T47D mice did not have a normal distribution and the variances were heteroscedastic. Therefore, we corrected for the heteroscedasticity and non-normality by transforming the data from their original form to a logarithmic form. One way ANOVA was used to analyze the difference in PSA level between the tumors of norgestrel-stimulated and non stimulated T47D mice. The test was also used to compare the PSA levels in the different tissues of T47D mice.

Results

Tumors developed in the T47D and LNCaP mice 3–4 months after cell inoculation with tumor incidence greater than 90%. The growth rate of the tumors, once they appeared, was similar with a doubling time of approximately one week. Figure 1 shows the mean and SD for PSA levels in tumors dissected from control T47D bearing mice, 5- or 7-day norgestrel-stimulated T47D bearing mice, and LNCaP bearing mice. A very low PSA level was present in the T47D bearing mice that were injected with sesame oil (control), with a mean of 2.4 pg PSA/mg protein (SD = 1.5). This level was not significantly different from background levels. In contrast, elevated PSA levels were found in 5- and 7-day norgestrel-stimulated T47D mice, with the means and SDs of 47 ± 41 and 140 ± 123 pg PSA/mg protein, respectively. Clearly, norgestrel induced PSA production in T47D mice, when administered for either 5 or 7 days. The mean and SD for PSA levels in LNCaP mice were 4430 ± 2710 pg PSA/mg protein, a concentration that is ∼30–100 times greater than the PSA levels in the stimulated T47D mice (Figure 1). Background PSA levels were detected in the muscle, liver, and kidneys from both

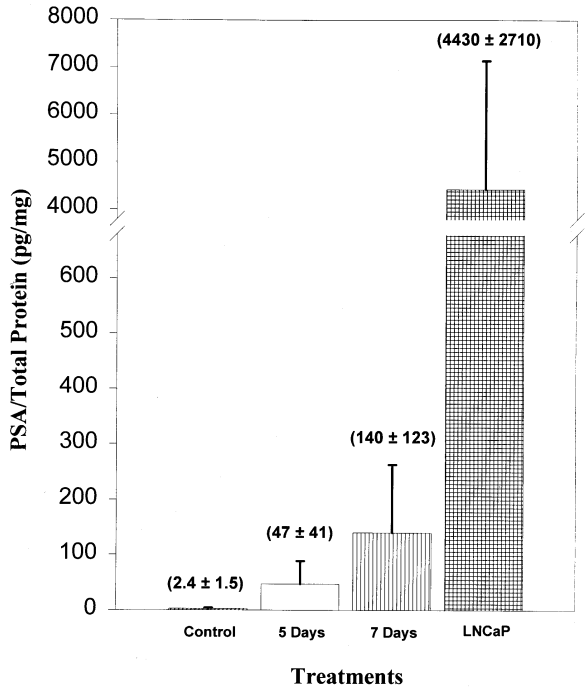

Figure 1. Mean and SD for PSA concentration (pg PSA/mg protein) in tumors from control T47D mice (4 tumors), 5-day norgestrel-stimulated T47D mice (4 tumors), 7-day norgestrel-stimulated T47D mice (5 tumors), and LNCaP mice (12 tumors).

control and norgestrel-stimulated T47D mice. However, the normal tissues from LNCaP mice had PSA levels that were 10–20% of those detected in the tumors (data not shown).

When analyzing the tumor samples from the four different groups of mice, a large SD was observed in all treatment groups (Figure 1). The large SD might be due to both intra- and inter-tumor variability in PSA levels. Thus, both sources of variability were examined in each treatment group. A large variability in PSA level was observed among tumors from individual T47D mice in the same treatment group (Figure 2). The mean PSA level in the 5-day norgestrel-stimulated mice ranged from 9.3 to 102 pg PSA/mg protein. In the 7-day norgestrel-stimulated mice, the PSA mean level ranged from 37 to 353 pg PSA/mg protein. Furthermore, when analyzing different samples from a single tumor, we found intra-tumor heterogeneity in the different samples, as seen by the large SD for the individual mice in Figure 2. Inter- and intra-tumor heteroge-

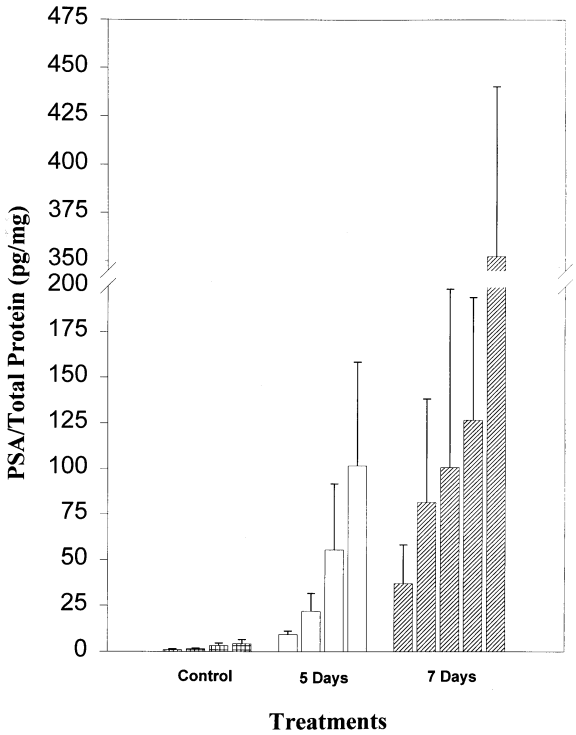

Figure 2. Intra- and inter-tumor variability in PSA concentration in T47D mice. Each bar represents a single mouse. The mean and SD for PSA concentration for each tumor were obtained by analyzing 3 different samples from a single tumor. These levels are the same levels for which group means are shown in Figure 1.

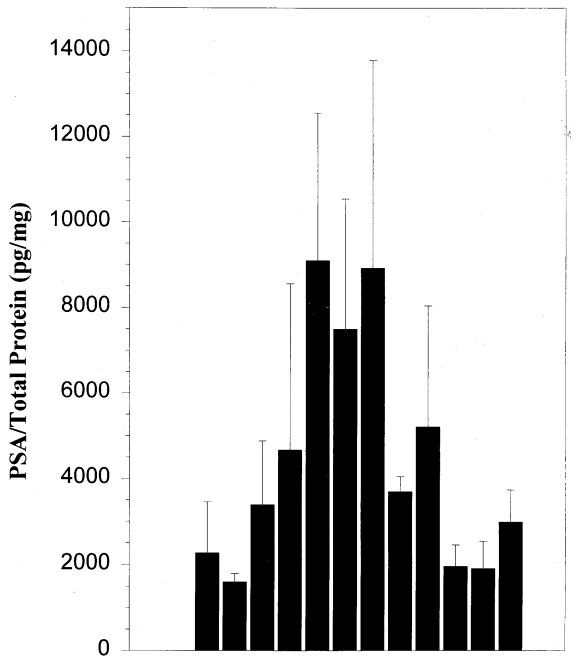

Figure 3. Intra- and inter-tumor variability in PSA concentration in LNCaP mice. Each bar represents a single mouse. The mean and SD for PSA concentration for each tumor were obtained by analyzing 3 different samples from a single tumor. These levels are the same levels for which group means are shown in Figure 1.

neity both contribute to the large SD seen in Figure 1.

Tumors from different mice inoculated with LNCaP cells also had variable levels of PSA, ranging from $\sim$ 1600 to 9000 pg PSA/mg protein. When analyzing 3 different samples from the same tumor, intra-tumor variability in PSA levels was observed in these mice, as seen by the large SD for most tumors in Figure 3. Furthermore, this intra-tumor heterogeneity was observed in immunohistochemically stained sections from LNCaP mice, as shown in Figure 4. Thus, the inter- and intra-tumor heterogeneity seen in norgestrel-stimulated mice was also observed in prostate tumors.

Discussion

In vitro experiments showed that steroid hormones, including progestins and androgens, can induce the human breast cancer cell-line T47D to produce PSA [14]. In the present investigation, an *in vivo* model for breast cancer PSA induction was established using SCID mouse xenografts. Elevated PSA levels were observed in T47D tumors in mice that were stimulated with norgestrel for either 5 or 7 days. However, T47D tumors in control mice that were injected with the vehicle only, showed low PSA concentrations that were close to background levels (Figure 1). Tumors from the control mice had a statistically significant difference in PSA levels compared to the tumors from the stimulated T47D mice ($p < 0.001$). Furthermore, PSA production in the norgestrel-stimulated T47D mice was tumor-specific since elevated PSA levels were only found in the tumors. In contrast, background PSA levels were present in the plasma, muscle, liver, and kidneys of these T47D mice as well as in all tissues of control mice (data not shown). Tumors of control and norgestrel-stimulated T47D mice did not show a difference in growth rate over the period of the stimulation.

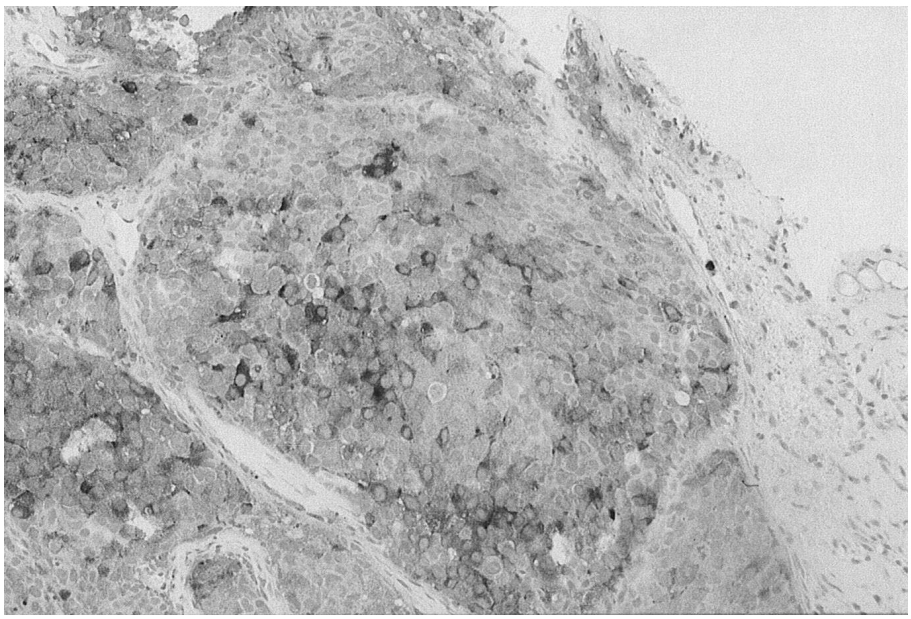

Figure 4. Immunohistochemical staining of tumor sections from LNCaP mice. The darker areas represent clusters of cells that are positive for PSA while the lighter areas represent cells that do not contain PSA. The magnification is × 250.

The tumors of LNCaP mice allowed a direct comparison of the PSA levels in this model to those produced in T47D mice after norgestrel stimulation. Tumors of LNCaP mice had PSA concentrations that were ∼ 30–100 times greater than the PSA concentrations in the norgestrel-stimulated T47D tumors. These results parallel observations of others who showed that the PSA concentrations in most human breast tumors are much lower than the PSA concentrations found in human prostate cancer [1, 8]. The normal tissue from LNCaP mice had PSA levels that were 10–20% of those detected in the tumors (data not shown). It appears high PSA concentrations in LNCaP tumors caused elevated levels in the blood, contributing to elevated PSA levels in the normal tissues.

Yu et al. [8] analyzed 1275 breast cancer cytosolic extracts and observed a large variability in the PSA concentration in the tumors. The PSA levels in their study varied from 10 to 100,000 pg PSA/mg protein and the PSA levels in the population were not normally distributed. In the present study, a large inter-tumor variability in PSA concentration was found in the norgestrel-stimulated T47D mice. The PSA concentrations in these mice ranged from 9 to 353 pg PSA/mg protein (Figure 2). Moreover, the concentrations of PSA in these mice were also not normally distributed. Therefore, there are similarities in PSA levels in the two populations, suggesting that the SCID mouse xenografts may be a useful system to investigate PSA production by breast tumors.

When analyzing different cross-sections from a single tumor, intra-tumor variability in PSA concentration was found in both T47D and LNCaP tumors, as observed by the large SD in Figures 2 and 3, and by the immunohistochemical staining of LNCaP tumors in Figure 4. Zarghami and Diamandis [17] found that the PSA immunoreactivity in human breast tumors is focal and restricted to clusters of cells. They explained these results by suggesting that not all tumor cells in PSA-positive tumors produce PSA, resulting in an uneven distribution of PSA in the tumors. However, it is also possible that distinct tumor samples from the LNCaP and norgestrel-stimulated T47D mice have different ratios of PSA-producing tumor cells to normal cells (e.g., stromal cells and lymphocytes) which do not produce PSA. The distribution of PSA in LNCaP tumors was also focal and restricted to clusters of cells, primarily in the cytoplasm of these cells, as seen in Figure 4. Sato et al. [18], in their recent stud-

ies of LNCaP tumors in SCID mice, observed a similar inhomogeneity in PSA distribution in stained tumor sections. Therefore, one could expect different samples from the same tumor to display different PSA levels, resulting in tumor heterogeneity.

The heterogeneity in PSA concentration in breast tumors can be attributed to factors such as androgen levels [19] and growth factors [20–23], which affect PSA synthesis and expression. However, the SCID mouse population was 'homogeneous' due to homozygosity and the identical number of tumor cells that were inoculated. Thus, one would not expect varying androgen and growth factor levels in the different mice. Progesterone receptors were also implicated in PSA production [14]. High progesterone receptor levels were detected in the tumors of both the control and the norgestrel-stimulated T47D mice. Limited data showed some qualitative association between PSA levels and progesterone receptor levels in control and 5-day stimulated T47D mice. However, low estrogen receptor levels were found in both control and norgestrel-stimulated T47D mice (data not shown). The high progesterone receptor levels relative to the estrogen receptor levels suggest that the former may be more important than the latter in affecting PSA synthesis. A similar conclusion has been recently reported by Zarghami et al. in their *in vitro* studies of steroid hormone regulation of PSA in T47D cells [24].

Using an ultrasensitive time-resolved immunofluorometric assay, Diamandis et al. [7] found that $\sim$ 30% of breast tumor cytosols contain immunoreactive PSA. Their results suggested PSA to be an independent favorable prognostic marker for some breast cancer subgroups [10]. The goal of clinical prognostic markers is to provide information that will allow physicians to choose a specific and beneficial therapy for each patient [25]. Currently, prevention of breast cancer is not feasible because many associated factors are endogenous and thus difficult to manipulate. Therefore, the only way to reduce mortality is through early diagnosis and administration of effective treatment.

Due to the heterogeneity of breast cancer, physicians use prognostic markers to identify patients who are at high risk for disease recurrence [11]. Although a variety of prognostic markers are available, these markers are not very sensitive and specific, making the classification of patients into subgroups inaccurate. Thus, it is quite difficult to identify patients who may benefit from adjuvant treatment, and selection of patients for appropriate therapy remains difficult and confusing [25, 26]. The present mouse xenograft model may be a valuable tool for studying the response of breast tumors to adjuvant therapy based on the PSA concentration. Furthermore, this model can contribute to the development and screening of novel therapies for a large subgroup of breast cancer patients.

More studies are necessary to determine the physiological role of PSA in the female breast to appreciate its importance as a prognostic marker for breast cancer. Recent findings suggest PSA to act as a negative growth regulator in hormone-dependent breast cancer [27]. In the future, classification of breast cancer patients based on the PSA concentration in the tumors, may aid in selecting patients for alternative/additional therapy.

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