**Title:** Progesterone Receptor Isoform Influence on Human Breast Tumor Growth and Hormone Treatment

**Abstract:**
Many postmenopausal women receive progestins in conjunction with estrogens as part of hormone replacement therapy. These women have a slightly higher risk of developing breast cancer than those receiving estrogen alone. Progesterone mediates its affects through two known forms of the progesterone receptor, PR-A and PR-B. We aim to study how progesterone, via either PR-A or PR-B, influences estrogen-stimulated tumor formation and growth. This is important since the two forms have been shown to have different effects on estrogen-stimulated pathways. We employ the female "nude" mouse, which lacks a complete immune system, as a model to study human breast tumor growth and response to hormone treatment. These mice are ovariectomized, have circulating levels of hormones similar to postmenopausal women, and serve as good hosts for growth of implanted human tumor cells. Using this model, we grow human breast cancer cell lines maintained in culture into solid breast tumors by direct injection of the tumor cells into the host. We replace either estrogens, progestins, or both via hormone implants in experimental animals and can thus control the dose and duration of treatment. The goal of our studies is to determine the effects of either PR-A or PR-B on estrogen-dependent growth of human breast tumors, and on the response of these tumors to endocrine therapies, specifically those involving progestins.

**Keywords:** progesterone, progesterone receptor, estrogen, estrogen receptor
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INTRODUCTION

Estrogen Receptors (ER) and Progesterone Receptors (PR) are both members of the same family of nuclear transcription factors and are coexpressed in many of the same target tissues. There is significant cross-talk between the estrogen and progesterone signaling pathways. Approximately two thirds of ER positive breast tumors that undergo endocrine therapy are also PR positive. How PR influences the outcome of therapies for ER positive tumors is unknown. Complicating this is the fact that PR exist as two naturally-occurring isoforms termed PR-A and PR-B, and these isoforms have been shown to have differential effects on ER-mediated transcription. Interestingly, PR positive primary human breast tumors have greatly varying ratios of PR-A:PR-B. In normal breast tissue and most breast tumors and cell lines, PR are under the regulation of estrogen, which makes it difficult to separate ER and PR effects. One exception are T47Dco human breast cancer cells, which are ER positive but constitutively express both PR-A and PR-B, independent of estrogen regulation. This makes them an excellent model for studying PR action. We have previously isolated a PR negative T47D subline (T47D-Y) and engineered them to express either PR-A (T47D-YA) or PR-B (T47D-YB). As outlined under this grant, we have established a model in which these cell lines can be grown into solid tumors as xenografts in female athymic nude mice. Growth of tumors in vivo occurs in a strictly estradiol-dependent manner. Preliminary data show that the PR status, regardless of ligand, affects estradiol-dependent tumor growth. In particular, cells expressing PR-A grew significantly smaller tumors, on average, than cells expressing no PR or PR-B. These data were presented at two international conferences as found in Appendices i and ii. An original article describing these data in detail is in press in Breast Cancer Research and Treatment. A copy of this publication is attached to this report as Appendix iii.
Results of experiments proposed in DAMD17-01-1-0508 are provided in detail in a submitted manuscript entitled "Progesterone Receptors A and B Differentially Affect the Growth of Estrogen-Dependent Human Breast Tumor Xenografts" provided as Appendix iii, pages 12-30. Briefly, specific aims and results are discussed below.

Establishment of ideal experimental conditions for growing T47D human breast cancer cells into solid tumors in vivo in female nude mice. Conditions for growing T47D tumors were successfully determined. Parameters such as cell number, suspension matrix, hormone delivery, etc. were carefully determined. The detailed procedures can be found in the reference section of Appendix iii, pages 14-15. Results of these studies can be found in Appendix iii, pages 12-30.

Treatment of established tumors with steroid hormones. Treatment of established tumors with estrogens (estradiol and tamoxifen) and progestins (progesterone and RU486) were accomplished by both using commercially available pellets and silastic hormone implants. Detailed information on steroid delivery can be found in Appendix iii, pages 14-15 & 24-25.

Creation of ER-alpha and ER-beta positive cells that will subsequently be used for tumor growth in vivo. These experiments were not carried out because of earlier data establishing that 1) all of the T47D cell lines used in these studies are ER-alpha positive and ER-beta negative, and 2) each of these cell lines was responsive to estradiol-stimulated growth on account of their expression of ER-alpha. It was therefore not necessary to stably integrate ER-alpha into these cells, and consequently ER-beta was not needed in addition.
KEY RESEARCH ACCOMPLISHMENTS

1. **Successful establishment of a model in which to grow T47D human breast cancer cells *in vivo* in nude mice.**
   In order to accomplish any of the goals of the proposed studies, it was necessary to first create a model system. The protocol for *in vivo* growth of T47D breast cancer cells was successfully established based on previously models established for other breast cancer cell lines such as MCF7. All experimental details for this procedure can be found in Appendix iii, pages 14-15.

2. **Creation of a system in which to study multiple hormone treatments on established T47D tumors: successful hormone delivery and confirmation of circulating blood levels.**
   Most breast cancer cells and breast tumors require specific hormonal stimulation for growth. In the case of T47D cells, chronic estradiol stimulation is necessary. This was provided by commercially available estradiol pellet implants. However, delivery of other steroids such as tamoxifen and progesterone were also needed. We adapted a silastic steroid hormone pellet procedure that efficiently delivered hormones into the blood stream of the experimental animals chronically for 6-10 weeks. The details of these procedures are provided in Appendix iii, pages 14-15. Circulating levels of estradiol are provided on page 24.

3. **Discovery that Progesterone Receptor isoform status significantly affects estrogen-dependent growth of T47D breast tumors.**
   The major finding of these studies, once the model was established, was that Progesterone Receptor isoform expression significantly impacted estrogen-dependent growth of T47D breast cancer cells in to solid tumors. Specifically, tumors that expressed PR-A (T47D-YA and T47D_c0) grew significantly smaller on average than tumors expressing PR-B only (T47D-YB) or no PR (T47D-Y). These results comprise the bulk of the submitted manuscript attached as Appendix iii, pages 12-30.
REPORTABLE OUTCOMES

Data compiled under the aims of this grant were presented at two scientific conferences:

Keystone Symposia on Nuclear Receptors, April 13-19th, 2002, Snowbird, Utah. The published abstract entitled “Role of PR-A vs. PR-B in Estrogen-Dependent Human Breast Tumor Growth” can be found in Appendix i, page 10.


Experiments completed under this grant are embodied in a manuscript entitled “Progesterone Receptors A and B Differentially Affect of Estrogen-Dependent Human Breast Tumor Xenografts” in press in Breast Cancer Research and Treatment, attached as Appendix iii, pages 12-30.
CONCLUSIONS

Several conclusions were drawn from these studies.

1. **T47D human breast cancer cells can be successfully grown into solid tumors in female nude mice.** This required testing several experimental procedures to optimize the conditions. Details of this procedure can be found in Appendix iii, pages 14-15.

2. **Growth of T47D tumors requires chronic estradiol stimulation, which can be efficiently delivered via either commercially available or silastic pellets.**

3. **Progesterone Receptor status affects the growth rate of estradiol-stimulated tumors.** Four cells lines with different PR status (PR negative, PR-A only, PR-B only, and both PR-A and PR-B) were compared. Cell lines expressing PR-A grew significantly smaller tumors than those expressing PR-B. See results section in Appendix iii for details (pages 15-17) and Figures 2 and 4, pages 27 and 29.
REFERENCES

Please see reference section in Appendix iii, pages 20-22.
Role of PR-A vs. PR-B in Estrogen-Dependent Human Breast Tumor Growth

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Approximately two thirds of ER positive human breast tumors that respond to endocrine therapy are also PR positive. However, PR positive tumors contain varying ratios of the two naturally-occurring receptor isoforms, PR-A and PR-B. PR-A and PR-B have different functions in vivo including differential effects on estrogen mediated actions in target tissues. To study how each PR isoform influences tumor growth, we have developed a model system in which human T47D breast cancer cells expressing either PR-A or PR-B are grown into solid tumors in ovariectomized female nude mice. Growth of tumors in vivo occurs in a strictly estradiol-dependent manner. Our data show that PR status, independent of ligand, affects estradiol-dependent tumor growth. In particular, cells expressing PR-B grow significantly larger tumors, on average, than cells expressing PR-A. This occurs with independently derived PR expressing cell lines and despite the fact that PR-A and PR-B containing cells have approximately equal population doubling times in culture. Addition of both estrogen and progesterone at initial tumor formation did not alter growth patterns. Differences in growth of established tumors were observed, however, between PR-A and PR-B containing tumors treated with either tamoxifen or the antiprogestin RU486. These experiments will help determine which types of tumors may be the best candidates for hormone therapies. This work was supported by DAMD BC996108 and the Cancer League of Colorado.
Differential Estradiol-Dependent Gene Expression in PR-A vs. PR-B Containing Human Breast Tumor Xenografts
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The two isoforms of progesterone receptors (PR), PR-A and PR-B, are expressed at varying ratios in estrogen receptor (ER) positive human breast cancers, and may potentially affect the outcome of endocrine therapies. We have used ER positive T47D human breast cancer cells that express PR-A exclusively or PR-B exclusively to grow solid tumor xenografts in ovariectomized female nude mice. Under chronic estradiol stimulation necessary for tumor growth, PR-A tumors grew consistently smaller than PR-B tumors; this occurred in the absence of PR ligand. We hypothesized that PR-A was specifically repressing ER regulated genes, analogous to that observed in vitro, except in a ligand-independent manner. To identify specific genes whose expression levels were affected by the presence of PR, we performed Affymetrix® gene microchip analyses (Human Genome U95Av2) on RNA isolated from estradiol-treated PR-A and PR-B expressing tumors. Affymetrix and Genespring® software was used to identify a subset of genes which were differentially expressed between the two samples in multiple experiments. Of the 59 genes consistently differentially regulated, 48 were expressed at higher levels in PR-B containing tumors, whereas 11 were expressed at higher levels in PR-A containing tumors. We will present data on specific genes may be important in mediating the differential growth difference between the two types of tumors (PR-A vs. PR-B). We conclude that the PR isoform ratio in primary breast tumors is important and should be considered clinically.
Running Title: Progesterone Receptors Affect Breast Tumor Growth

Article type: report

Progesterone Receptors A and B Differentially Affect the Growth of Estrogen-Dependent Human Breast Tumor Xenografts

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Summary

Sixty-70% of all primary human breast cancers are estrogen-dependent and express both estrogen (ER) and progesterone receptors (PR). Whereas expression of the two naturally occurring PR isoforms, PR-A and PR-B, is close to equimolar in normal human tissues, the ratio of the two receptors varies extensively in tumors. This is important since the two PR are functionally distinct and have differential repressor effects on ER. The PR isoform content may, therefore, affect the outcome of endocrine therapies targeted at ER. Study of PR isoforms is difficult because the two receptors are co-expressed in cells under estradiol stimulation. We have engineered four sets of T47D human breast cancer cells that, independent of estrogen: i) express only PR-A; ii) express only PR-B; iii) are PR-negative; or iv) contain both PR isoforms. Each of these cell lines was grown into solid tumors in nude mice in a strictly 17β-estradiol-dependent manner. Results show, first, that PR-A expressing cells grow into tumors that are approximately half the size of PR-B expressing tumors, and second, that the reduced growth of PR-A tumors occurs in the absence of PR ligand. Tamoxifen treatment preferentially inhibited the growth of PR-A tumors, whereas PR-B tumors were unaffected. Thus, PR are not just passive markers of functional ER; the prevalence of PR-A or PR-B may differentially influence tumor phenotype.

Key Words: Progesterone Receptors, Breast cancers, Estrogens, Ligand-Independence, Xenografts

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Introduction

Receptors for progesterone (PR) and estrogen (ER) are co-expressed in female breast and reproductive tissues and their ligands influence one another’s actions. Normally, PR are induced by estradiol, which leads to expression of two different isoforms transcribed from the same gene [1]. The two receptors – PR-A and PR-B – are identical except that PR-B have 164 additional amino acids at the N-terminal B upstream segment (BUS), which contains a strong activation function [2, 3]. As a result, liganded PR-B are usually much more potent transcriptional activators than PR-A, both in vitro [4] and in vivo [5]. The two PR isoforms are co-expressed in progesterone target tissues. Selective inactivation of PR-A or PR-B in the mouse has established distinct roles for the two isoforms in vivo, with PR-A playing a more dominant role in the reproductive tract and PR-B playing a larger role in normal breast differentiation [6, 7]. In contrast to PR, the two ER, termed ER-α and ER-β, are transcribed from separate genes and have overlapping but distinct tissue distributions [8-11]. Gene inactivation studies of ER-α and ER-β in mice have established that ER-α is the dominant mediator of estradiol effects in reproductive tissues and the mammary gland [12, 13].

Total PR are routinely measured in breast cancers as a guide to therapy because they are presumed to be markers of a functional estrogen/ER signaling pathway. However, PR positive tumors, irrespective of treatment, also have a better prognosis than PR negative tumors for unknown reasons. While current clinical assays measure total PR and cannot distinguish between the isoforms, we now know that the two PR are expressed unequally in breast cancers. Two detailed studies have demonstrated that the ratio of PR-A to PR-B varies considerably among tumors [15, 16]. This observation is important since the two PR are functionally distinct and differentially inhibit ER-mediated transcription, with PR-A having stronger ER transrepressor properties than PR-B [17, 18]. Thus, it is likely that the PR status of receptor positive tumors influences, not only their phenotype, but also the outcome of endocrine therapies targeted at ER. We describe here a model that demonstrates PR isoform influence on estrogen-dependent tumor growth and treatment.

Several ER positive human breast cancer cell lines have been widely used to study hormonal effects on tumor growth [19]. Among these, T47D cells are unique because they express equimolar levels of PR-A and PR-B, in a constitutive and estrogen independent manner [20, 21]. We previously isolated a PR negative subline (T47D-Y) of T47D cells that were used as recipients to stably reintroduce either PR-A or PR-B expression vectors [2]. The resulting cell lines, called T47D-YA and T47D-YB, have been used to study the independent function of each PR isoform in response to progesterone [5, 22].

Here we describe a new experimental model in which the four T47D cell lines, each with a different PR content, are grown into solid tumors in 17β-estradiol supplemented, ovariectomized nude mice. We find that tumors expressing PR-A are half the size of tumors expressing PR-B, suggesting that PR-A preferentially inhibit estrogen-dependent growth. This occurs in a ligand-independent manner, since progesterone is not required. Tamoxifen preferentially inhibits the growth of PR-A expressing tumors; PR-B expressing tumors are tamoxifen resistant. Together, the data support the concept that differential PR isoform expression, in the absence of progesterone, can influence estrogen-dependent tumor growth characteristics and the outcome of tamoxifen treatment. Thus, PR are more than passive markers of ER function. We propose that future clinical assays need to identify the PR isoform that is dominant in a tumor.
Materials and Methods

Human breast cancer cell lines

Construction of T47D-Y (PR negative), T47D-YA (PR-A expressing), and T47D-YB (PR-B expressing) cell lines has been described previously [2]. T47D and MCF-7 cells were maintained in culture in MEM supplemented with 5% FBS. T47D-YA and T47D-YB cells were additionally supplemented with 200 μg/mL G418. Population doubling (PD) times were determined by seeding cells at 1 x 10⁶ per 75 mm flask, collecting and counting cells 72 hours later, and calculating PD (h) using the equation: \( 72^\text{ln}2/\text{ln}\text{final cell number}/1 \times 10^6 \). Additional cell lines stably expressing PR-A (T47D-A1, T47D-A11) or PR-B (T47D-B3, T47D-B9) were constructed by transfection of T47D-Y cells with PR-A and PR-B expression vectors that dually express the neomycin resistance gene (pCDNA3.1, Invitrogen, San Diego, CA). Colonies were selected by supplementing media with 500 μg/mL G418 and screened for PR expression by immunoblotting (see below).

Experimental animals and human breast tumor xenograft model

Animal procedures were approved by the University of Colorado Institutional Care and Use Committee. Ovariectomized female athymic nu/nu mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN) at 5-6 weeks of age. Animals were anesthetized with Avertin (2.5% 2,2,2-Tribromoethanol in sterile 1x PBS) at 16 μL per g. Animals were implanted with commercial 60-day slow release pellets containing 1.7 mg of 17β-estradiol (Innovative Research, Sarasota, FL) or with silastic pellets (see below). T47D cells were collected, counted, and suspended in MEM at a concentration of 25 x 10⁶ cells/mL. 5 x 10⁷ cells in a total volume of 400 μL (200 μL cell suspension plus 200 μL reduced growth factor Matrigel (Becton-Dickinson, Bedford, MA) were injected into experimental animals on both the left and right flanks, using a 26 gauge needle. Tumors were monitored weekly for growth by measuring length (l) and width (w) with a digital caliper. Tumor area was calculated by the formula lwxw. At termination of the experiment, mice were euthanized by CO₂ asphyxiation, and tumors excised and weighed. In some cases uteri were removed and weighed. Statistical analyses were performed using GraphPad Software (San Diego, CA). When comparing two groups, t tests were used (unpaired or paired depending on the data sets). When comparing three or more groups one-way ANOVA followed by a Tukey post-test was used.

Silastic hormone pellets and serum hormone levels

Silastic hormone pellets were prepared using a protocol adapted from Guzman et al [23]. Silastic tubing (1.96 mm ID x 3.18 mm OD, Dow Corning, Midland, MI) was sectioned into 1 cm pieces and sterilized by autoclaving. One end was sealed with aquarium cement and allowed to dry. All hormones were purchased from Sigma (St. Louis, MO). For 17β-estradiol pellets, powder was weighed and added to cellulose at a ratio of 2:13 g. Cellulose (placebo), tamoxifen, and the estradiol/cellulose mixture were each pulverized into fine powder with a mortar and pestle and packed into silastic tubing with sealed Pasteur pipettes. The open ends of the pellets were then sealed with aquarium cement. Each pellet contained approximately 15 mg of powder. Pellets were incubated in 0.9% NaCl at 37°C for 24 h prior to implantation. Single pellets were used for the duration of the experiments. Whole blood was collected from animals at time of euthanasia and serum separated by centrifugation at 9000 g for 5 min at 4°C. Serum levels of 17β-estradiol were determined by chemiluminescence microparticle immunoassay (CMIA, Abbott Laboratories, North Chicago, IL).
**Immunostains and immunoblots**

For immunostaining, tumors were fixed in 10% buffered formalin and paraffin embedded. 5 μm sections were processed and stained (Histology Core Lab, UCHSC) with an antibody to PR (PgR, Ventana, Tucson, AZ). For protein extraction, tumors were frozen in liquid nitrogen and pulverized into a fine powder. TEDG (10 mM Tris pH 7.4, 1 mM EDTA pH 7.4, 1 mM DTT, 10% glycerol) + 0.3 M KCl + protease inhibitors (Roche, Indianapolis, IN) were added at 200 μL per 100 mg of tissue and samples were homogenized at 4°C in a Dounce tube with a glass pestle. The homogenates were incubated on ice for 1 h and separated by centrifugation at 100,000 g for 30 min at 4°C. KCl was removed from supernatants by passing samples over Q25 sephadex columns. Protein extracts from cultured T47D cells were prepared in the same manner from collected cell pellets. Protein concentrations were determined by Bradford assay. 100 μg of total protein from each sample were resolved on a 7.5% SDS-PAGE gel, transferred to nitrocellulose and probed with monoclonal antibodies to PR (AB-52 and B-30) [24] or to ER (AB-15, Neomarkers, Fremont, CA).

**Results**

**Human breast tumor xenograft models to study the role of PR**

The independent study of PR-A and PR-B in estrogen-dependent breast tumors is difficult since all models co-express both PR isoforms under estrogen control. We developed T47D human breast cancer cell models that differ in their PR isoform expression in an estrogen independent manner. T47DCO cells express equal levels of the two PR in the absence of estradiol [21]. The PR-negative T47D-Y subline has been in continuous culture over six years, and was used to create stable PR-A (T47D-YA) or PR-B (T47D-YB) expressing cell lines [2]. To test tumor estrogen dependence and define their growth characteristics, T47DCO (PR-A + PR-B positive) and T47D-Y (PR-negative) cells were grown for 6-weeks in intact cycling females, or in ovariectomized mice implanted with 1.7 mg 17β-estradiol pellets (Figure 1). T47DCO and T47D-Y cells were implanted on opposite flanks of the same experimental animals. Surprisingly, intact cycling animals did not support tumor growth. However, substantial tumors grew in the ovariectomized animals supplemented with estradiol. Circulating levels of 17β-estradiol in cycling and estradiol-supplemented mice are summarized in Table 1, and show that the pellets supply physiological or high physiological hormone levels. We speculate that continuous estradiol levels, rather than cycling levels, are necessary to sustain T47D tumor growth. Subsequent experiments were done in estradiol-supplemented, ovariectomized mice. Interestingly, larger tumors grew from the PR negative cells than the PR positive cells (Figure 1), suggesting that PR expression can suppress estrogen-dependent tumor growth even in the absence of progesterone.

The PR isoform content of T47D tumors influences their estrogen dependent growth

To determine whether both PR isoforms exhibit this growth suppressive property, the four cell
lines, T47D-Y, T47Dco, T47D-YA, and T47D-YB, each with different PR isoform content, were implanted into ovariectomized nude mice. For direct comparison, tumors were grown in pairs on opposite flanks of the same animals: T47D-YA and T47D-YB were paired, as were T47D-Y and T47Dco. Experimental animals were further divided into untreated (control) or estrogen treated (0.1 mg 17β-estradiol pellets) groups (see Table 2 for details). Tumor growth was monitored weekly for six weeks and mean tumor area/week are plotted in Figure 2A. None of the four cell lines grew appreciably in control animals, with tumor areas starting and remaining at approximately 25 mm². In the presence of estradiol, however, the four T47D cell lines, regardless of PR status, grew substantially. On average, in the same animals, the tumor area of T47Dco (57.4 mm²) was smaller than that of T47D-Y (76.7 mm²). Similarly, T47D-YA tumor area (49.2 mm²) was smaller than that of T47D-YB (78.7 mm²).

At the end of six weeks, the animals were euthanized and tumors were removed and weighed. Figure 2B depicts masses of the four T47D tumor types from control and estradiol-treated animals. Data were compiled from three separate experiments in which a total of 120 tumors were analyzed, with 30 tumors per cell type. Tumor masses and statistical significance are summarized in Table 2. In ovariectomized untreated controls, cells formed into solid sheets but failed to grow – few reached 100 mg in mass. Even in these tumors, however, average masses differed significantly (p<0.01 to 0.001) depending on the PR content. Control T47D-Y tumors had greater mass (74.4 mg) than control T47Dco tumors (29.7 mg); and control T47D-YB (67.2 mg) tumors had greater mass than control T47D-YA tumors (34.4 mg).

Continuous estradiol treatment generated tumors with approximately four-fold greater mass than controls with, interestingly, a similar pattern emerging. Estradiol-stimulated T47D-Y tumors (291.4 mg) had greater mass than the paired T47Dco tumors (187.1 mg), and T47D-YB tumors (281.7 mg) had twice the mass as the paired T47D-YA tumors (141.6 mg). The distribution of tumor masses was such that T47Dco tumors never grew greater than 300 mg and T47D-YA tumors did not exceed 250 mg. In contrast T47D-Y and T47D-YB grew multiple large tumors ranging from 300-600 mg in mass. The fact that in each case, larger tumors were paired with smaller tumors on the same mice, suggests that the smaller tumors grew poorly because of some intrinsic property of the cells and not the animals. Of interest is the fact that the smaller tumors always contained PR-A.

The tumors retain appropriate PR-isoform expression
To document that appropriate PR expression was maintained in the four T47D tumor types after six weeks of estradiol-stimulated growth in vivo, mice were inoculated with 5 x 10⁶ T47D cells and implanted with 17β-estradiol pellets, as above. Figure 3A shows paraffin sections of solid tumors stained with an antibody that recognizes both PR. Sections from T47D-Y tumors were PR negative, indicating that chronic estradiol treatment in vivo does not reactivate expression of the endogenous PR gene. Sections from T47Dco, T47D-YA, and T47D-YB tumors all stained positive for PR. All four tumors had a poorly differentiated phenotype with cells growing as sheets, and with an absence of glandular differentiation.

To determine whether the appropriate PR isoform specificity was maintained, protein extracts from the 6-week estradiol stimulated tumors (T) were immunblotted and compared to extracts from the parental cell lines grown in culture (C) (Figure 3B). Both the T47D-Y cell lines and corresponding tumors were PR negative, T47Dco cell lines and tumors expressed both isoforms of PR, 120 kD PR-B and 94 kD PR-A, as expected. T47D-YA tumors expressed only PR-A, and T47D-YB tumors only PR-B, analogous to the cell lines from which they were derived. These data show that all four T47D cell sublines grow into tumors that retain the appropriate PR isoform expression. Immunoblots were also probed with an antibody to ER-α, and the four T47D tumor sets contain ER levels equivalent to those seen in MCF-7 tumors (not shown). Immunoblots were negative for ER-β expression (not shown).
PR-A inhibit growth of estrogen-dependent T47D tumors in a ligand-independent manner

The studies in Figure 2 suggested that compared to PR-B, PR-A expression suppresses tumor growth. To rule out the possibility that this is due to trivial clonal variations between T47D-YA and T47D-YB cells, unrelated to the receptors, additional PR-positive cell lines were engineered from the PR negative T47D-Y cells. Two new PR-A expressing cell lines (T47D-A1 and T47D-A11) and two new PR-B expressing cell lines (T47D-B3 and T47D-B9) were constructed. The immunoblot in Figure 4A shows their PR status. Parental T47D-Y cells are PR negative and wild-type T47Dsc cells express both PR-A and PR-B. The three PR-A cell lines, -YA, -A1 and -A11 express only PR-A; the three PR-B cell lines, -YB, -B3 and -B9 express only PR-B.

These new cell lines were grown into estrogen-stimulated solid tumors in vivo. Three sets of PR-A and PR-B expressing cells were grown on opposite flanks of the same animals: T47D-YA were paired with T47D-YB, T47D-A1 were paired with T47D-B9; and T47D-A11 were paired with T47D-B3. Figure 4B compares the growth of all PR-A expressing tumors (n = 30) and all PR-B expressing tumors (n = 30). Although all tumors were the same size initially, PR-B expressing tumors grew larger than PR-A expressing tumors during the first six weeks of the experiment. As depicted in Figure 4C, the average mass of PR-A tumors (132.4 ± 49.3 mg) was significantly lower than that of PR-B tumors (234.6 ± 92.8 mg). Figure 4D shows the final masses of individual tumors from each of the three pairs, and their average mass. In each of the three pairs, PR-A expressing tumors were, on average, smaller than PR-B expressing ones regardless of the independently derived cell line from which they arose. This clearly shows that growth suppression is related to the presence of PR-A and unrelated to clonal variations among the cell lines. The masses of 60 individual tumors of the 30 pairs are shown in Figure 4E. Recall that each pair was grown in the same animal. With the exception of 3 pairs, in which PR-A and PR-B tumors had the same mass, in the other 25 pairs, PR-B mass was always greater than PR-A. Not a single example exists of a PR-A tumor larger than its PR-B partner. Interestingly, no significant differences in the growth rate of PR-A and PR-B expressing T47D cells have been observed in culture (PD 33.2 ± 1.2 and 32.8 ± 1.6 h, respectively). This underscores the importance of in vivo models to study certain hormone-dependent growth parameters.

PR-A expressing tumors are preferentially inhibited by tamoxifen

PR and ER status are used as markers of hormone responsiveness to treatments like tamoxifen. We therefore asked whether the tamoxifen responsiveness of these estrogen-dependent tumors was influenced by the PR isoforms present therein. MCF-7 human breast cancer cell xenografts were used as controls, since their growth is known to be tamoxifen inhibited [25]. MCF-7, T47D-YA and T47D-YB tumors were first established for 3 weeks on opposite flanks of 2 mg estradiol treated animals. The animals were then given either placebo (15 mg) or tamoxifen (15 mg) implants for an additional 5 weeks, while estradiol was continued. Efficiency of tamoxifen delivery was assessed by measurement of mean uterine masses at the end of treatments (Table 3). In estrogen-treated animals, tamoxifen significantly reduced uterine size compared to placebo, indicating that sufficient tamoxifen was delivered. Figure 5A compares the growth of estrogen-stimulated tumors treated with either placebo or tamoxifen. The growth of MCF-7 tumors was significantly (p < 0.05) inhibited by tamoxifen. Growth of T47D-YA tumors was also inhibited by tamoxifen in a statistically significant (p < 0.05) manner. However, the growth of T47D-YB tumors was unaffected by tamoxifen.

Tumor masses following treatments are summarized in Figure 5B. MCF-7 tumors treated with tamoxifen were significantly smaller than their placebo treated counterparts. Similarly, mean final masses of tamoxifen treated T47D-YA tumors were significantly lower than the placebo controls. This was observable despite the small size of the T47D-YA tumors. T47D-YB tumors were unaffected by tamoxifen. These experiments were performed three times with similar results. We conclude that tumors expressing PR-A are more sensitive to tamoxifen than tumors expressing PR-B, independent of progesterone.
Discussion

Overview. Approximately one-third of breast cancers are hormone dependent. To identify this subset, assays of total ER and PR have been in use for many years [26, 27]. Presence of ER alone is much less useful as a predictive marker (likelihood of response to hormone treatments is 35-50%) than if PR are also present (likelihood of response is 80-75%). Additionally, presence of PR in tumors indicates a good prognosis [28]. The role of PR has been somewhat puzzling, however, because most hormonal treatments – tamoxifen for example – are targeted at ER rather than at PR. Thus it has been assumed that PR do not play a direct role in the biology of the tumor. Rather, because PR are upregulated by estradiol, they have been presumed to mark the soundness of the ER response pathway. Here we describe the surprising finding that PR can play a direct role in the phenotype of an estrogen-responsive tumor model, and do so in a progesterone-independent manner. This explains in part, why PR are important in breast cancers even if they are not direct targets of treatment by progestational agents.

Additionally, when clinical assays for PR in breast cancers were first developed, the existence of two isoforms was in dispute [29]. Evidence for PR-A and PR-B is now solidly established, and much work has shown that the two receptors are functionally different [30, 31]. However, clinical assays have not caught up with the extensive molecular and biological information developed in the last decade regarding the two PR, so that to date, oncologists are still restricted to treating patients based on assumptions and assays developed in the 1980’s. The studies we describe here indicate that information about the PR-A vs. PR-B status of a tumor may be critical to understanding the hormone responsiveness and biological properties of that tumor; hence critical to clinical decision-making.

The models. We have developed ER positive, estrogen-dependent human breast tumor xenografts models that express no PR, PR-A, PR-B, or both. All the cells were derived from the identical parental T47Dco line. Therefore, the models allow us to assess the independent contribution of each PR isoform to the phenotype of the otherwise genetically identical, estrogenized tumor background. This cannot be done in other models in which PR are under estrogen control, making it impossible to dissect out tumor responses to estrogens, from responses due to presence of PR. Additionally, because in other models the two PR isoforms are co-expressed, it is impossible to dissect out the contributions of each. Using our new models, we show i) that the presence of unliganded PR in a tumor inhibits estrogen-dependent tumor growth (Figure 1) and ii) that this inhibition is due mainly to the influence of the PR-A isoform (Figures 2, 4). As a corollary, tamoxifen sensitivity appears to be restricted to tumors expressing PR-A (Figure 5).

Ligand independent actions of PR. One of the surprising findings in this study is that the estrogen inhibitory effects of PR do not require progesterone. There are some precedents for this. Chicken (c) PR-A are activated in a ligand-independent manner by cAMP and epidermal growth factor [32, 33]. In addition to cPR, several other nuclear receptors can be activated by dopamine through D1 receptors [34], and dopamine placed directly into the third ventricle of the brain increases female rat sexual behavior in a progesterone-independent but PR dependent manner [35]. Attempts to demonstrate such effects for human PR have been unsuccessful. However, using breast cancer cells in which human PR are under control of an inducible system, we recently showed by microarray profiling that expression of unliganded PR leads to regulation of a definable set of endogenous genes [36]. Interestingly, three times more genes were uniquely regulated by unliganded PR-A than by unliganded PR-B. This was surprising, since the opposite is the case for regulation by liganded PR [5]. Given this, it is possible that the well-defined ER inhibitory effects of PR-A [17, 18] are in part mediated in a ligand-independent manner. This would explain why PR-A expressing cells grow smaller tumors in an estrogenized environment despite the absence of progesterone (Figure 2).
Two PR isoforms in the breast and breast cancers. That human breast cancer cells co-express both PR isoforms is well established [37, 38], but newer data demonstrate that this is also the case clinically. Graham et al [15] assessed the PR-A to PR-B ratio by immunoblotting 202 PR-positive tumors. A large number of tumors (45%) had a non-equimolar PR-A:PR-B ratio. Of these, the majority (32%) overexpressed PR-A, and in 25%, PR-A exceeded PR-B by more than 4-fold. A recent study by this group [39] demonstrates that loss of coordinate PR-A to PR-B expression is an early event in tumor formation. In the normal human breast the two receptors are equimolar, but unequal ratios are evident even in early proliferative disease without atypia, and there is increasing heterogeneity with disease progression. As a result, half of invasive tumors have lost coordinate PR isoform expression, and in 80% of these, there is an excess of PR-A.

What is the biological consequence of this dysregulation? Little is known about this. An analysis of 53 tumors shows that equimolarity of PR isoform levels is associated with a well-differentiated phenotype [16]. In this study, PR-A excess was associated with ErbB2/neu overexpression, a higher tumor grade, and other features of poor differentiation and an aggressive phenotype. Similarly, in cultured human breast cancer cells, over-expression of PR-A is associated with enhanced motility, loss of adherent properties, and morphological changes indicative of poor prognosis and heightened metastatic potential [39]. Thus, what little information is available, suggests that PR-A excess is particularly harmful. How does that jibe with our observations that PR-A expressing tumors are smaller than their PR-B counterparts?

This question requires extensive study, but raises the possibility that the tumors may be smaller, but more aggressive. There are important clinical examples of breast cancers that, despite their small size, have a high metastatic potential. Kleer et al [40] recently characterized Rhoc expression in benign and malignant breast disease in association with small tumor size. Rhoc is a member of the Ras superfamily of GTPases and a transforming oncogene for human mammary epithelial cells. Its over-expression results in a highly motile and invasive phenotype, and the authors suggest that it may be a marker of small breast cancers with high metastatic potential. Interestingly, in our hands, expression of Rhoc is upregulated by unliganded PR-A but not by unliganded PR-B[3]. Taken together, current data indicate that PR-A over-expressing tumors, which, depending on cut-off values represent one-third to two-thirds of PR-positive tumors, may be especially aggressive even if small, and would require aggressive therapeutic interventions. Currently, there are no clinical assays that identify the PR isoform specificity of breast tumors.

3 B. M. Jacobsen, personal communication.
Acknowledgments

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Figure legends

Figure 1. PR-negative tumors grow to a larger size than PR-positive tumors. Growth of PR-negative T47D-Y (PR-) and PR-positive T47DCC (PR+) tumors was measured in intact (cycling) and in ovariectomized nude mice supplemented with 1.7 mg 17β-estradiol pellets (ovx + E). All animals were 5-6 weeks of age at initiation of the experiment. Mean tumor areas plus SE are shown. Data represent an n of 10 per condition. Tumors grown in ovx + E animals are significantly larger than tumors in cycling animals, p<0.0001; and PR-negative tumors are significantly larger than PR-positive tumors, p<0.001.

Figure 2. In the absence of progesterone, PR expression affects estrogen-dependent growth of T47D cell lines in vivo. (A), Growth of T47D cell lines into solid tumors in vivo. Ovariectomized nude mice were injected subcutaneously on the left and right flank with T47D-Y or T47DCC cells, or with T47D-YA or T47D-YB cells, and implanted with either placebo (control) or 17β-estradiol (E) pellets as described. Tumors were measured for six weeks with a digital caliper. Mean tumors areas including SE are shown. Estradiol-stimulated tumors were each significantly larger than untreated controls, p<0.0001. (B), Final masses of T47D tumors from control and 17β-estradiol (estrogen) treated animals. Both the distribution of individual tumors (open symbols) and mean masses (bar) plus SE are shown. T47D-YA and T47DCC tumors were significantly smaller than T47D-Y and T47D-YB tumors (p<0.001 and p<0.01, respectively). Numerical data, sample numbers, and statistics are detailed in Table 2.

Figure 3. T47D cell-derived tumors retain appropriate PR isoform expression. (A), Paraffin sections of T47D-Y, T47DCC, T47D-YA and T47D-YB tumors were stained for PR expression with an antibody that recognizes both PR-A and PR-B (magnification 40x). (B), Immunoblot of protein extracts from T47D-Y, T47DCC, T47D-YA, and T47D-YB cell lines (C) and corresponding tumors (T). Protein extracts (100 µg) were resolved on a 7.5% SDS-PAGE gel, transferred to nitrocellulose, and probed with anti-PR antibodies AB-52 and B-30 [24]. Protein bands were visualized by enhanced chemiluminescence. Approximate molecular weights are indicated.

Figure 4. Estrogen-dependent growth of T47D tumors is inhibited by unliganded PR-A. (A), Immunoblot showing PR expression in the new cell lines T47D-A1, -A11, -B3 and -B9 compared to the existing cell lines (T47D-YA, -YB). (B), 17β-estradiol stimulated growth comparing all PR-A expressing (n = 30) and all PR-B expressing tumors (n = 30). Mean areas plus SE are shown. *PR-A expressing tumors grew smaller by 6 weeks, p<0.0001. (C), Final masses of all PR-A expressing and all PR-B expressing tumors. Bars indicate the mean masses of PR-A tumors (132.4 ± 49.3 mg) and PR-B tumors (238.6 ± 92.8 mg). *PR-A tumors were significantly smaller, p<0.0001. (D), Masses of PR-A and PR-B expressing tumors grown side by side on the same animals. Pairs include T47D-YA and T47D-YB (YA, YB; n=12), T47D-A1 and T47D-B9 (A1, B9; n=10), and T47D-A11 and T47D-B3 (A11, B3; n=8). Mean mass (bar) plus SE are shown. (E), Masses of individual tumor pairs (n = 30 pairs) showing variation among mice. Each pair was grown on opposite flanks of one animal. In no case is a PR-A tumor larger than its PR-B partner. PR-A are significantly smaller, paired t test, p<0.0001.

Figure 5. Tamoxifen preferentially inhibits growth of PR-A expressing tumors. (A), Ovariectomized mice were injected with MCF-7, T47D-YA or T47D-YB cells and supplemented with 2 mg 17β-estradiol pellets as described. After three weeks (arrow), mice were implanted with a second hormone pellet, containing placebo or tamoxifen (15 mg each) and tumors were followed for five additional weeks. Mean areas plus SE are shown. For MCF-7 tumors, data represent an n of 6. For T47D-YA and T47D-YB tumors, 20 placebo and 18 tamoxifen treated
animals were pooled from three separate experiments. *MCF7 tumors treated with tamoxifen grew smaller than controls, p<0.05. **T47D-YA tumors treated with tamoxifen grew significantly smaller than untreated controls, p<0.05. (B) Final mean masses plus SE of MCF-7, T47D-YA and T47D-YB tumors from placebo and tamoxifen treated animals. ***Tumors from tamoxifen treated animals had significantly smaller masses than tumors from placebo treated animals, p<0.05. Note the scales are half for T47D-YA because of their small size.

Table 1. Circulating levels of 17β-estradiol in experimental mice

<table>
<thead>
<tr>
<th>animal</th>
<th>Estradiol pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>128 ± 56</td>
</tr>
<tr>
<td>Ovx</td>
<td>31 ± 6</td>
</tr>
<tr>
<td>Ovx + 1.7 mg E²</td>
<td>262 ± 66</td>
</tr>
<tr>
<td>Ovx + 2 mg E³</td>
<td>139 ± 42</td>
</tr>
<tr>
<td>Ovx + 2 mg E + 15 mg Tam⁸</td>
<td>119 ± 24</td>
</tr>
</tbody>
</table>

Blood was collected from three experimental animals per condition after six to eight weeks of treatment with hormone pellets.

* Commercial pellets (Innovative Research).
$ Silastic pellets containing 2 mg 17β-estradiol or 15 mg tamoxifen. Serum levels of 17β-estradiol were determined by CMIA (Abbott laboratories).

Mean serum estradiol plus SD are shown.
Table 2. Mean mass (mg) of T47D cell tumors with different PR status

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PR</th>
<th>Control*</th>
<th>n</th>
<th>Estrogen*</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>T47D-Y</td>
<td>none</td>
<td>74.4 ± 39.4</td>
<td>14</td>
<td>291.4 ± 128.5</td>
<td>30</td>
</tr>
<tr>
<td>T47D-0Ca</td>
<td>A &amp; B</td>
<td>29.7 ± 10.9</td>
<td>14</td>
<td>187.1 ± 51.6</td>
<td>30</td>
</tr>
<tr>
<td>T47D-YA</td>
<td>A</td>
<td>34.4 ± 11.7</td>
<td>15</td>
<td>141.6 ± 44.9</td>
<td>30</td>
</tr>
<tr>
<td>T47D-YB</td>
<td>B</td>
<td>67.2 ± 19.2</td>
<td>15</td>
<td>281.7 ± 114.1</td>
<td>30</td>
</tr>
</tbody>
</table>

T47D cells were grown into tumors in female ovariectomized nude mice supplemented with placebo (control) or estrogen (1.7 mg 17β-estradiol) pellets. Tumors were excised and weighed after growth for six weeks in vivo. Mean masses, SD, and sample numbers (n) are shown.

* T47D-0Ca (p<0.001) and T47D-YA (p<0.01) tumors are significantly smaller than T47D-Y and T47D-YB control tumors, when compared by ANOVA using a Tukey post test.

* Estradiol-stimulated T47D-0Ca (p<0.01) and T47D-YA (p<0.001) tumors were significantly smaller than T47D-Y and T47D-YB tumors, when compared by ANOVA using a Tukey post test.

Table 3. Uterine mass (mg) in response to hormone treatment in experimental mice

<table>
<thead>
<tr>
<th>Hormone treatment</th>
<th>Uterine mass</th>
<th>n</th>
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<tbody>
<tr>
<td>Normal</td>
<td>74.0 ± 15.7</td>
<td>9</td>
</tr>
<tr>
<td>Ovx</td>
<td>21.7 ± 4.4</td>
<td>4</td>
</tr>
<tr>
<td>Ovx + E</td>
<td>119.0 ± 28.7*</td>
<td>16</td>
</tr>
<tr>
<td>Ovx + E + placebo</td>
<td>111.2 ± 17.9</td>
<td>10</td>
</tr>
<tr>
<td>Ovx + E + Tam</td>
<td>48.3 ± 10.6*</td>
<td>9</td>
</tr>
</tbody>
</table>

Uteri were removed and weighed from experimental animals after 6-8 weeks of treatment. Normal animals had intact ovaries. Ovx: ovariectomized; E, 17β-estradiol, Tam, tamoxifen. Mean mass plus SD are shown. Experimental groups were compared to each other by ANOVA using a Tukey post test.

*Significantly larger compared to normal and ovx, p<0.001

*Significantly smaller compared to ovx + E + placebo, p<0.001
Figure 1

- ovx + E
- cycling

area (mm²)

weeks

PR−, PR+
Figure 2

A

**T47D<sub>co</sub>**
- E
- control

**T47D-Y**
- E
- control

**T47D-YA**
- E
- control

**T47D-YB**
- E
- control

B

**control**

**estrogen**

mass (mg)

<table>
<thead>
<tr>
<th>CO</th>
<th>TY</th>
<th>YA</th>
<th>YB</th>
</tr>
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<tbody>
<tr>
<td>0</td>
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<td>300</td>
</tr>
<tr>
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<td>600</td>
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<table>
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</tr>
<tr>
<td>500</td>
<td>600</td>
<td>700</td>
<td>800</td>
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</tbody>
</table>
Figure 3

A

T47D-Y

T47D

T47D-YA

T47D-YB

B

<table>
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<tr>
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<th>T47D&lt;sub&gt;CO&lt;/sub&gt;</th>
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<th>T47D-YB</th>
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</thead>
<tbody>
<tr>
<td>C</td>
<td>T</td>
<td>C</td>
<td>T</td>
</tr>
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</table>

120 kD

94 kD
Figure 5