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# The Proliferative Response to p27 Down-Regulation in Estrogen Plus Progestin Hormonal Therapy is Lost in Breast Tumors

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## Abstract

Increased proliferation and breast cancer risk has been observed in postmenopausal women receiving estrogen (E) + progestin hormone replacement therapy (HRT). Progestin action is mediated through two progesterone receptor (PR) isoforms, PRA and PRB, with unique transcriptional activity and function. The current study examines hormonal regulation of PR isoforms in the normal postmenopausal human breast and the mechanism by which progestins increase proliferation and breast cancer risk. Archival benign breast biopsies from postmenopausal and premenopausal women, and luminal breast tumor biopsies from postmenopausal women, were analyzed for regulation of PRA and PRB expression by E and E+medroxyprogesterone acetate (MPA). In the postmenopausal breast without HRT, PRA and PRB expression was decreased compared to the premenopausal breast. Both E (n = 12) and E+MPA (n = 13) HRT in the postmenopausal breast were associated with increased PRA and PRB expression, increased nuclear cyclin E expression, and decreased nuclear p27 expression compared to no HRT (n = 16). With E+MPA HRT, there was a further decrease in nuclear p27 and increased Receptor Activator of NF-kappa B Ligand (RANKL) expression compared to E-alone HRT. In luminal breast cancers, E+MPA HRT (n = 6) was also associated with decreased nuclear expression of the cell cycle inhibitor p27 compared to E HRT (n = 6), but was not associated with increased proliferation. These results suggest that p27 mediates progestin-induced proliferation in the normal human breast and that regulation of this proliferative response by E+MPA is lost in breast tumors.

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## Introduction

Progesterone (P) and synthetic progestins have been implicated in the etiology and progression of breast cancer in both animal models and the human breast [1]. In the human postmenopausal breast, hormone replacement therapy (HRT) with the conjugated equine estrogen (E) + the progestin medroxyprogesterone acetate (MPA) increases breast cancer risk over E alone [2–6]. Following the Women's Health Initiative findings on E +MPA HRT in 2002, a decline in HRT use was associated with decreased breast cancer incidence [7,8]. However, in both the Women's Health Initiative randomized trial [9] and the E3N cohort [10], a significantly elevated risk of breast cancer continued, even after stopping HRT.

The progesterone receptor (PR) mediates the action of P and synthetic progestins in the mammary gland (reviewed in [11]) and exists as two isoforms, PRA and PRB. The full-length isoform PRB, and the truncated isoform PRA, are encoded from the same gene and mRNAs. Ligand-activated PRs dimerize (A:A, B:B, and A:B) and localize to the nucleus where they repress or activate PR-target genes. *In vitro* studies using human breast cancer cell lines have shown that PRA and PRB have unique transcriptional activity and function [12]. Thus, relative

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expression of PRA and PRB is an important determinant of progestin action in the human breast. In normal premenopausal breast epithelium, PRA and PRB are co-expressed at similar levels and altered PR isoform (i.e., PRA) expression has been observed in the progression of breast cancer [13–16]. Alterations in PR isoform expression may be due to transcriptional regulation or may also be due to increased turnover of active ligand-bound PRB [17]. Total PR rather than PR isoform expression is usually measured in the clinical context, and while it is well-established that E regulates overall PR expression [18], hormonal regulation of individual PR isoforms PRA and PRB *in vivo* in the human breast has not been studied.

E+MPA HRT in normal breast is associated with increased proliferation, increased epithelial content, and increased breast density [19]. In addition, E+MPA HRT has been linked to increased risk of more aggressive breast cancers that are associated with a higher rate of breast cancer death [20]. Proliferation of breast epithelium is highest during the luteal phase of the menstrual cycle when endogenous P level is highest [19,21–24]. Treatment of cultured primary normal human breast cells with P increased proliferation through activation of pathways involved in DNA replication licensing [25]. While it is clear that progestins in the postmenopausal breast influence proliferation and breast cancer risk, the detailed mechanism of progestin action *in vivo* in the normal, intact human breast remains poorly understood.

In this study, the effect of HRT on expression of PRA, PRB and potential downstream regulators of progestin action was examined in normal postmenopausal breast tissue samples and in luminal breast tumors from postmenopausal women who had received hormonal therapy with either E alone or E+MPA.

## Materials and Methods

### *Normal Premenopausal and Postmenopausal Human Breast Samples*

Archival human breast samples were used from a cross-sectional, observational study carried out to study breast tissue from cycling, premenopausal (n = 10) and postmenopausal (n = 31) women undergoing surgical breast biopsy at Lansing, Michigan area hospitals. Of the postmenopausal women, 25% of those with no HRT experienced a surgical menopause; 75% of those with E HRT; 7.7% of those with E+MPA HRT. In the original tissue collection, biopsies were carried out to diagnose suspicious palpable lesions upon physical exam or suspicious mammographic densities. The protocol for fresh tissue collection of samples was approved by the University Committee on Research Involving Human Subjects and Institutional Research Review Boards of the participating hospitals; written informed consent was obtained from each patient. Biopsies collected for study were kept on ice, then fixed in 3.7% buffered formalin within 2 h of surgery for paraffin embedding.

Profiles of the study populations of postmenopausal women are summarized in Table 1. Postmenopausal women were defined as those who had experienced 12 consecutive months of amenorrhea, had a bilateral oopharectomy at least 1 year before biopsy, or were 55 years of age or older. Subjects were placed into one of three categories: (1) no HRT, defined as not having taken hormones for 1 year before surgery; (2) E-alone; or (3) E+progestin. HRT subjects were defined as those taking hormones for at least 3 months continuously up to the day of surgery. All hormones were taken on a continuous, daily basis. Subjects had taken E in one of two forms: conjugated equine estrogens (dose, 0.3–2.5 mg; n = 11) or micronized estradiol (dose, 0.5 mg; n = 1). The progestin taken was medroxyprogesterone acetate (MPA; dose, 2.5–5 mg; n = 13), which was taken in combination with conjugated equine estrogens (n = 11) or micronized estradiol (n = 2). Herein, all types of estrogens are referred to as E. The specific progestin used in all studies was MPA, so all E+progestin HRT is referred to as E+MPA HRT.

**Table 1.** Postmenopausal Breast Sample Subject Characteristics

| Characteristic | No HRT | E | E+MPA |
|---|---|---|---|
| Number of subjects | n = 16 | n = 12 | n = 13 |
| Mean age (y) (range) | 63.7 ± 12.0 (41-77) | 63.9 ± 9.7 (43-79) | 62.1 ± 9.7 (54-88) |
| Body mass index (kg/m2) | 27.7 ± 5.1 | 27.3 ± 6.8 | 27.5 ± 6.8 |
| Time on HRT (y) (range) | n/a | 15.8 ± 8.9 (4-34) | 7.4 ± 4.4 (3-20) |
| Menopausal status (%) | | | |
| Natural | 75.0% | 25.0% | 92.3% |
| Surgically induced | 25.0% | 75.0% | 7.7% |
| Reproductive History | | | |
| Nulliparous (%) | 12.5% | 8.3% | 0% |
| Parous (%) | 87.5% | 91.7% | 100% |
| Mean no. pregnancies (range) | 3.9 ± 2.4 (2-7) | 2.9 ± 1.3 (1-5) | 3.2 ± 1.3 (2-7) |
| Mean no. deliveries (range) | 2.7 ± 1.6 (1-6) | 2.8 ± 1.3 (1-4) | 2.9 ± 1.2 (2-6) |
| Age at 1st delivery (y) (range) | 22.2 ± 4.0 (16-31) | 20.4 ± 2.9 (16-26) | 22.1 ± 3.4 (17-28) |

Premenopausal subjects were divided into two categories depending on the phase of the menstrual cycle: (1) follicular, days 1–14; or (2) luteal, days 15–28.

### *Postmenopausal Human Breast Cancer Samples*

Archival formalin-fixed paraffin-embedded blocks of human breast cancer samples were obtained from Sparrow Hospital in Lansing, Michigan. These samples were unrelated to the samples of normal breast tissue examined in this study. The protocol for obtaining archival samples and identifying relevant samples was approved by the Human Research Protection Program at Michigan State University and the Institutional Research Review Board of Sparrow Hospital. Medical record analysis was performed at Sparrow Hospital to select samples originating from postmenopausal women receiving E HRT (n = 6) or E+MPA HRT (n = 7). Information was not available on whether any of the tumor samples were derived from women that had surgically induced menopause. After selection of samples to examine, de-identified archival breast tumor samples (Table 2) were analyzed at Michigan State University. Pathology reports on all the tumor samples chosen indicated that they were ER and PR positive, suggesting a luminal subtype for all breast tumors. Breast tumors from women that had not received HRT were not available for this study.

### *Immunofluorescence*

Sections (5 μm) were mounted onto 3-aminopropyl-triethoxysilane (Sigma, St. Louis, MO)-coated cover slips, and assayed by immunofluorescence on nonserial sections. The number of tissue samples assayed varied because, in some cases, there was not enough tissue for all assays, or some tissue sections only contained lobules or ducts, and not both. Sections were deparaffinized, rehydrated, and subjected to antigen retrieval (20 min at 121°C, 16 p.s.i. in citrate buffer (pH 6.0)) prior to immunofluorescent detection.

Single antibody immunofluorescence labeling was performed with rabbit polyclonal anti-cyclin E (1:50, Santa Cruz, sc-481), anti-p27 (1:250, Santa Cruz, sc-528), anti-RANKL (1:500, Novus), anti-Amphiregulin (Areg) (1:100, Neomarkers, Ab-1 RB-257-P1), and mouse monoclonal anti-Ki67 (1:100, Dako, MIB1) antibodies. Primary antibodies were recognized by appropriate secondary antibodies conjugated to Alexa 488 (Molecular Probes, Invitrogen). Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen).