**Microenvironment and Immunology**

**Progestosterone Receptor Signaling in the Microenvironment of Endometrial Cancer Influences Its Response to Hormonal Therapy**

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Abstract

Progestosterone, an agonist for the progesterone receptor (PR), can be an efficacious and well-tolerated treatment in endometrial cancer. The clinical use of progestosterone is limited because of the lack of biomarkers that predict hormone sensitivity. Despite its efficacy in cancer therapy, mechanisms and site of action for progestosterone remain unknown. Using an in vivo endometrial cancer mouse model driven by clinically relevant genetic changes but dichotomous responses to hormonal therapy, we show that signaling through stromal PR is necessary and sufficient for progestosterone antitumor effects. Endometrial cancers resulting from epithelial loss of PTEN (PTENKO) were hormone sensitive and had abundant expression of stromal PR. Stromal deletion of PR as a single genetic change in these tumors induced progesterone resistance indicating that paracrine signaling through the stroma is essential for the progesterone therapeutic effects. A hormone-refractory endometrial tumor with low levels of stromal PR developed when activation of KRAS (PTENKO/Kras) was coupled with PTEN-loss (PTENKO/Kras). The innate progesterone resistance in PTENKO/Kras tumors stemmed from methylation of PR in the tumor microenvironment. Add-back of stromal PR expressed from a constitutively active promoter sensitized these tumors to progesterone therapy. Results show that signaling through stromal PR is sufficient for inducing hormone responsiveness. Our findings suggest that epigenetic derepression of stromal PR could be a potential therapeutic target for sensitizing hormone-refractory endometrial tumors to progesterone therapy. On the basis of these results, stromal expression of PR may emerge as a reliable biomarker in predicting response to hormonal therapy. Cancer Res; 73(15); 4697–710. ©2013 AACR.

Introduction

Typically, antagonists of steroid receptors are exploited in the therapy for hormonally regulated carcinomas (1–3). Endometrial cancer is unique in that agonistic actions of progestosterone exert antitumor effects. Uterine cancers are the most prevalent gynecologic malignancy in the western world with a rising incidence in the United States (4). Most uterine tumors arise from the endometrium, a hormonally regulated cell layer composed of epithelium and stroma. Endometrioid carcinomas, characterized by crowded disorganized epithelial glands with few intervening stroma, are the most common subtype of these tumors (5). Some of these cancers originate from excess proliferation induced by imbalances in estrogen and progestosterone. Activation of oncogenes or loss of tumor suppressors initiates other tumors that are fueled by hormonal imbalances (6). Although hormonal therapy is successfully used in treatment and chemoprevention of breast (1, 2) and prostate adenocarcinomas (3), it is less widely embraced in therapy for endometrial cancers.

In the normal endometrium, prolonged exposure to unopposed estrogen can cause endometrial hyperplasia and cancer (7–9). Administration of high-dose progesterone induces thinning of the normal endometrial lining (10). Given that progestosterone causes atrophy of the normal endometrium, it is administered clinically as a single agent in the therapy for endometrial cancer. Despite five decades of clinical use, the antitumor mechanisms and site of action for progestogene therapy remain unknown. Molecular mechanisms underlying progestosterone resistance or sensitivity are also poorly understood.

Progestosterone is well tolerated, easily administered, and has minimal side effects. Subsets of patients respond to progestosteone, whereas others have progression of disease while on hormonal therapy (11). Response rates to progestosterone range from 11% to 50% in primary and recurrent disease (12).
Despite its efficacy, hormonal therapy is not commonly administered in treatment of endometrial cancer primarily because patients with hormone-sensitive or -resistant disease cannot be prospectively identified. Standard therapy for endometrial cancer involves removal of reproductive organs, at times coupled with adjuvant radio- or chemotherapy (13). Although this approach may be curative, it causes loss of fertility and can induce life-long side effects. Patients with metastatic disease often succumb to the cancer despite aggressive treatments (14). Discovery of reliable biomarkers that predict responsiveness to progesterone therapy and molecular mechanisms that dictate hormone sensitivity or resistance could broaden the application of hormonal therapy to patients with endometrial cancer.

A challenge in endometrial cancer research is the perception of the endometrium as a homogeneous tissue. The endometrium is composed of epithelium and stroma, two distinct cell types with unique functions and responsiveness to steroid hormones (15, 16). To study contributions of each cell type in tumor initiation and progression, we established an in vivo endometrial regeneration model from dissociated epithelial and stromal populations (17). This model provides a unique tool for induction of concomitant but separate genetic changes in these two compartments (17) an experimental approach not achievable with existing endometrial cancer models. Here, we used our dual compartment regeneration system as an in vivo preclinical platform for testing responsiveness to hormonal therapy in endometrial tumors generated from clinically relevant genetic changes. Tumors resulting from epithelial loss of PTEN were exquisitely progesterone sensitive, whereas tumors resulting from activation of KRAS concomitant with PTEN-loss were completely progesterone resistant. Using these endometrial cancer models with dichotomous responses to hormonal therapy, we show that signaling through stromal progesterone receptor (Pgr or PR) is necessary and sufficient for antitumor effects of progesterone therapy.

Materials and Methods

Animals

Wild-type (WT) C57BL/6, Pten^{fllox/lox} (C;129S4-Pten^{tm1Hwu/J}), Kras^{LSL-G12D+} (B6.129S4-Kras^{Jmu4T8}) and CB17Scid/Scid mice were from The Jackson Laboratory. Ptg^{fllox/lox} (PRCE) mice were from Dr. Luisa Inzela-Arispe (University of California, Los Angeles, Los Angeles, CA). Pten^{fllox/lox} Kras^{LSL-G12D+} mice were generated by crossing the Pten^{fllox/lox} background. Mice were maintained in accordance with University of California, Los Angeles (UCLA; Los Angeles, CA), Division of Laboratory Animal Medicine (DLAM) guidelines. All animal experiments were approved by the UCLA Animal Research Committee.

Lentivirus constructs and preparation

The Cre-RFP (17), Cre-GFP (18), and GFP (19) lentiviral constructs have been previously described. Generation of the hPr-GFP and inducible Cre-shPten-GFP constructs is outlined in Supplementary Methods. Lentivirus production, titering, and infection of cells were conducted as previously described (20).

Preparation of endometrial stroma and epithelia

Mouse neonatal uterine stroma and adult endometrial epithelial were prepared as previously described (17). Endometrial epithelial were isolated to purity by fluorescence-activated cell sorting (FACS) sorting as reported previously (21). To obtain adult endometrial epithelia, mouse uteri were dissected, cut into fragments, and incubated in 1% trypsin for 45 minutes at 4°C. Luminal epithelial were separated from underlying stroma with a fine forceps and digested with 0.8 mg/mL collagenase in Dulbecco’s Modified Eagle Medium (DMEM)/10% FBS with 0.5 mg/mL DNase for 1.5 hours at 37°C. Digested fractions were passed through 22-gauge syringes and filtered through 40-μm cell strainers to yield single-cell suspensions. Cells were stained on ice with shaking for 15 minutes for FACS. Endometrial epithelial marked by Trop1° CD90° CD45° CD31° Ter119° were FACS isolated using a BD FACSaria II flow cytometer. Antibodies used for FACS are listed in Supplementary Tables.

Tumor generation

Endometrial tumor generation was conducted as previously described (17). In some experiments endometrial epithelium or stroma were infected with lentivirus as described in Supplementary Methods. For all experiments, approximately 125,000 prepared epithelial and 200,000 stromal cells were mixed, resuspended in collagen (BD Biosciences; 354236), and dispensed into grafts. Endometrial grafts were implanted under the kidney capsule of oophorectomized CB17Scid/Scid mice and regenerated for 6 to 8 weeks with an estrogen pellet (60-day time release, 0.72-mg β-estradiol/pellet; SE-121 Innovative Research of America). Tumors generated with 100% efficiency using this 1:1 to 1:2 epithelial to stromal cell ratio.

Hormone therapy

An 8-week course of progesterone therapy was administered by subcutaneous implantation of a time release pellet (60-day time release, 100 mg progesterone/pellet; SP-131 Innovative Research of America). Placebo treatment in control mice was achieved by implantation of placebo pellets (60-day time release, 100 mg progesterone placebo/pellet; SC-111 Innovative Research of America). Estrogen pellets (60-day time release, 0.72-mg β-estradiol/pellet; SE-121 Innovative Research of America) were replenished at the start of progesterone therapy, unless otherwise noted. All surgical procedures were carried out under the UCLA DLAM regulations. Serum hormone levels in mice were verified using estradiol EIA and progesterone EIA kits (Cayman Chemical).

Immunohistochemistry

Immunohistochemistry was conducted as previously described (17) using antibodies listed in Supplementary Tables. To quantify expression of K67, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL), and cleaved caspase-3, 5 to 10 high-power fields of view were scored per sample and averaged.
Isolation of epithelia and stroma from regenerated tumors
PTENKO or PTENKO/Kras color-marked tumors were minced and digested in 1 mg/mL each of collagenase and dispase in DMEM/10% FBS with 5 µg/mL insulin and 0.5 mg/mL DNase for 2 hours at 37°C. Resulting cells were passed through a 22-gauge syringe to yield a single-cell suspension that was visualized and sorted using the BD FACSAria II.

Quantitative PCR
Quantitative PCR (qPCR) was conducted as previously described (21). RNA for qPCR was extracted from isolated tumor cell fractions using the AllPrep DNA/RNA Micro Kit (Qiagen). Data were normalized to the housekeeping gene Gapdh. Primers are outlined in Supplementary Tables.

Western blot analysis
Western blotting of isolated cell fractions was conducted as previously described (21) using antibodies outlined in Supplementary Tables.

Methylation-specific PCR and bisulfite sequencing
DNA extracted from isolated tumor cell fractions was analyzed by methylation-specific PCR and bisulfite sequencing using previously reported protocols (22) with minor modifications. Detailed descriptions and a list of the primers used are presented in Supplementary Methods.

Statistical analysis
Results are expressed as mean ± SD. To determine significance, comparisons were conducted using a two-tailed t test for groups of two- or one-way ANOVA with Tukey honestly significant difference test for three or more groups.

Results
Progesterone therapy effectively treated endometrial tumors resulting from cell autonomous loss of PTEN
Mutations in the PTEN tumor suppressor gene are reported in up to 79% of endometrioid endometrial cancers (23) where PTEN expression is lost predominantly in the tumor epithelium (24). Given the prevalence of this genetic change, defining whether PTEN status is a biomarker of response to hormonal therapy could be a valuable clinical tool. Results of retrospective clinical studies on this subject vary widely, with some studies reporting PTEN-loss as a positive predictor (25, 26), whereas others report loss of PTEN as a negative predictor of response to progesterone (27, 28). A problem in these studies is that tumors examined may contain other genetic alterations in addition to PTEN-loss. We hypothesized that tumors resulting from epithelial loss of PTEN, as a single genetic change, would be sensitive to hormonal therapy.
To address this hypothesis, the effects of progesterone therapy on PTEN-null tumors were examined using an in vivo model system (Fig. 1A). This model (17) mimics epithelial-specific loss of PTEN seen in human endometrial cancers (24) unlike existing eutopic mouse models where Pten is deleted in both the epithelium and stroma (29–31). Endometrial epithelia were FACS isolated from uteri of Pten-loxp/loxP mice and infected with lentivirus expressing red fluorescent protein (RFP) and Cre recombinase (17), resulting in deletion of Pten (Fig. 1A). These epithelia were combined with WT stroma and regenerated in the subrenal space of oophorectomized mice supplemented with estrogen via implantation of an 8-week time-release 17β-estradiol pellet (Fig. 1A; ref. 17). At 8 weeks, one mouse was sacrificed to confirm establishment of PTEN-null endometrioid endometrial tumors (PTENKO) that had a high proliferation index (Supplementary Fig. S1A and S1B). Given that endometrial cancer uncommonly occurs in patients with high estrogen levels (32), tumor-bearing mice were reimplanted with new estradiol pellets to maintain a hyper-estrogenic state. Simultaneously, half of these mice were implanted with progesterone pellets, whereas the other half were treated with placebo for 8 weeks (Fig. 1A). Measurement of serum hormone levels confirmed high circulating levels of estrogen and progesterone in progesterone-treated mice but only high levels of estrogen in the placebo group (Supplementary Fig. S1C). Large tumors were attached to the kidneys of placebo-treated mice (Fig. 1B, a and b), whereas only small cysts were found on kidneys of progesterone-treated animals (Fig. 1B, c and d). The histology of placebo-treated PTENKO tumors was endometrioid with epithelial-specific loss of PTEN (Fig. 1C, a and b and Supplementary Fig. S1D). Progesterone-treated PTENKO endometrial tumors resolved and remaining tissue was primarily a simple cyst lined with normal appearing PTEN-null epithelium (Fig. 1C, e and f and Supplementary Fig. S1D).

Hormone-mediated resolution of PTEN-null endometrial tumors is time-dependent but occurs efficiently in a short period
The time required for resolution of endometrial cancers treated with progesterone therapy varies widely (33). In some patients, tumors resolve within a few weeks of therapy, whereas others require more than 6 months of progesterone administration to show a clinical response (33). Many factors could contribute to this variation: dose and mode of drug administration, genetic heterogeneity in endometrial cancers, and variations in the patient’s endogenous hormonal milieu. An advantage in our model is the homogenous genetic background of tumors and the host mice bearing tumors. Also, we are able to pharmacologically control the experimental hormonal milieu. This model enables us to systematically investigate the response of PTENKO tumors to hormonal therapy.
We asked if hormonal resolution of tumors in our model is a kinetic process, occurring rapidly or incrementally.

To assess kinetics of tumor response, mice bearing PTENKO carcinomas were treated with progesterone for 2, 4, 6, and 8 weeks (Fig. 2A). Tumors were examined grossly and histologically at each time point (Fig. 2B). Proliferation of tumor epithelia was measured and quantified using Ki67 (Fig. 2C and Supplementary Fig. S2A), whereas apoptotic cell death was...
measured with TUNEL assay and expression of cleaved caspase-3 (Supplementary Fig. S2B). Progesterone therapeutic effects were observed as early as 2 weeks, evidenced by formation of cystic regions in the tumor (Fig. 2B, b, g, and l vs. a, f, and k) coupled with a significant decrease in proliferation (Fig. 2C and Supplementary Fig. S2Ab vs. S2Aa). Progressive tumor clearance was detected at 4 (Fig. 2B, c, h, and m) and 6 weeks (Fig. 2B, d, i, and n). Although the tumor proliferation plateaued between 2 and 6 weeks (Fig. 2C), apoptotic cell death increased significantly during this time period (Supplementary Fig. S2Bb–d and S2Bg–i). Complete tumor resolutions was observed at 8 weeks (Fig. 2B, e, j, and o). Cessation of progesterone therapy at this time point resulted in tumor recurrence in a hyper-estrogenic state (Supplementary Fig. S2C). Epithelial-specific PTEN-loss was verified in tissue harvested at all time points (Fig. 2B, p–t).

Effective tumor therapy requires a shift in the balance between cellular proliferation and cell death. We observed a biphasic response to progesterone in this kinetic experiment. In the first therapeutic phase cell proliferation ceased, whereas in the second phase cell death ensued, resulting in resolution of the remaining tumor cells. Findings here show that progesterone antitumor effects in PTEN-null endometrial tumors are quick but kinetic resulting from a combination of decreased proliferation and increased cell death.

Coadministration of estrogen was essential for progesterone-mediated antitumor effects in PTEN-null endometrial tumors

Although some patients with endometrial cancer have normal levels of circulating estrogen, many have a hyper-estrogenic
state resulting from a variety of causes (32). Thus far, we administered continuous estrogen during progesterone therapy to mimic a hyper-estrogenic state. Estrogen alone promotes progression of endometrial cancer; therefore, in clinical practice progesterone is administered without estrogen. To replicate standard practice, we tested the efficacy of progesterone without estrogen in therapy for PTENKO tumors.

Mice bearing PTENKO tumors were divided into two cohorts (Fig. 3A). In half the mice, existing estrogen pellets were removed and progesterone pellets were implanted (progesterone therapy alone; Fig. 3A). In the second group, the existing estrogen pellets were removed but new estrogen and progesterone pellets were implanted (estrogen and progesterone cotherapy; Fig. 3A). Before initiating treatment, establishment of pretherapy tumors was confirmed (Fig. 3B, a–c). Cotherapy with estrogen and progesterone resolved PTENKO endometrial tumors shown by development of cysts (Fig. 3B, e–g) with decreased epithelial proliferation compared with pretherapy.

Figure 3. Effective therapy with progesterone requires coadministration of estrogen. A, established PTENKO tumors were treated with (i) estrogen with progesterone cotherapy (n = 4) or (ii) progesterone therapy alone (n = 8). B, in the absence of estrogen, progesterone hormonal therapy failed to resolve PTENKO tumors. Establishment of tumor was confirmed before therapy (a–d). Resolution of tumors was observed with estrogen and progesterone cotherapy (e–h). Persistence of the tumor was noted with progesterone therapy alone (i–l). C, lower levels of stromal PR were detected in tumors treated with progesterone alone (c vs. a and b). Scale bars equal 100 μm except where noted.
tumors (Supplementary Fig. S3A). Surprisingly, PTENKO tumors treated with progesterone alone persisted (Fig. 3B, i–k) and had a proliferation index comparable with pretherapy tumors (Supplementary Fig. S3A). ER-α was expressed in the epithelia and stroma of all PTENKO tumors (Supplementary Fig. S3B). Notably, PTENKO tumors treated with progesterone alone had significantly diminished stromal PR (Fig. 3C, c) compared with estrogen and progesterone cotreated (Fig. 3C, b) and pretherapy counterparts (Fig. 3C, a). Abundant expression of epithelial PR was detected in all conditions (Fig. 3B (d, h, and l) and C).

Findings here show that coadministration of estrogen with progesterone is required in hormonal therapy for PTENKO tumors. Although in clinical practice progesterone is administered without estrogen, in fact many patients with endometrial carcinomas are often obese and have elevated levels of estrogenic estrogen due to the activity of aromatase in adipocytes (32, 34). Thus, in many patients progesterone is acting in a high estrogenic hormonal milieu similar to that used in our experiments. Differences in the hormonal milieu of patients with endometrial cancer may account for the wide variation in time required to achieve therapeutic responses (33).

Stromal deletion of PR is sufficient to convert a hormone-sensitive tumor to a hormone-refractory cancer

Despite its efficacy, the mechanisms of progesterone action and signaling in endometrial tumors have not been systematically investigated. Progesterone is the agonist for the PR (35). PR is expressed in the normal endometrial epithelium and stroma. With progression to cancer, expression of PR can become deregulated and lost in either cellular compartment (36). Given that PR is the primary target for progesterone, its expression has been examined as a predictor of response to hormonal therapy in endometrial cancer. In most studies, expression of PR correlated with better response to hormonal therapy, but some PR-negative tumors also respond to progesterone (37, 38). A problem in these studies is that PR was examined in tumor lysates or histologic sections without carefully assessing its epithelial and stromal expression (37, 38). It is unclear whether epithelial or stromal PR signaling mediates effects of progesterone therapy. We observed that PTENKO tumors became progesterone resistant with diminution of stromal PR (Fig. 3B (I) and C (c)). We hypothesized that progesterone therapeutic effects in endometrial cancer are mediated through PR signaling in the stroma of the tumor microenvironment.

To address this question, PR was selectively deleted in the stroma of PTENKO endometrial tumors. Neonatal PrloxP/loxP− (39) endometrial stromal cells were infected with Cre-expressing or control lentivirus marked with GFP and cultured for short term (Fig. 4A). Expression of Cre in these cells resulted in loss of PR-A and PR-B (Supplementary Fig. S4A). Combinations of FACSCalibulated PR-deleted (Cre-GFP) or PR WT (GFP) stroma with PTENKO epithelia were implanted in mice supplemented with estrogen throughout the course of this experiment (Fig. 4A and Supplementary Fig. S4B). After tumors were established, mice were treated with progesterone or placebo (Fig. 4A). In tumors with stroma expressing WT PR (GFP infected stroma), resolution of tumor was detected after progesterone treatment (Fig. 4B, a and b), whereas tumors persisted in placebo-treated controls (Fig. 4B, d and e). A significant drop in proliferation index was noted concomitant with tumor resolution with progesterone therapy (Supplementary Fig. S4C). Abundant expression of stromal PR was detected in this cohort (Fig. 4B, c and f). When PR was deleted in the stroma of PTENKO tumors (Cre-GFP–infected stroma), progesterone-treated tumors persisted and resembled placebo-treated counterparts (Fig. 4B, g and h vs. j and k). The proliferation index of epithelia in progesterone- and placebo-treated PTENKO PR-deleted tumors was equivalent (Supplementary Fig. S4C). Loss of PR in the stroma of these tumors was confirmed by immunohistochemistry (Fig. 4B, i and l). Our results show that one genetic change, deletion of stromal PR, was sufficient to convert hormone-sensitive PTENKO tumors to hormone-refractory endometrial cancers (Fig. 4B). Conversely, epithelial-specific deletion of PR in PTENKO tumors did not impact their response to progesterone therapy (Supplementary Fig. S4D).

These results show that signaling through stromal PR is essential for mediating antitumor effects of hormonal therapy in endometrial cancer. In developmental tissue recombination studies, stromal PR signaling decreased estrogen-mediated DNA synthesis in endometrial epithelia (40). During pregnancy, signaling through stromal PR inhibits endometrial epithelial proliferation to support fetal implantation (41). Our results show that signaling through stromal PR decreases epithelial tumor proliferation and induces apoptotic cell death in endometrial tumor tissue. We suspect that mediators of the antitumor effects of progesterone signaling from stroma are paracrine-secreted growth factors.

Endometrial tumors driven by concomitant loss of PTEN and Kras activation are refractory to progesterone therapy

Epithelial Pten loss as a single genetic change was a positive predictor of response to hormonal therapy in our model. Activation of KRAS is a common mutation coexisting with PTEN-loss in 21% of endometrial cancers (23). We asked if the addition of KRAS activation to PTENKO tumors would alter the susceptibility of resulting endometrial cancers to hormonal therapy.

Endometrial epithelia from Pten+/−/loxP KrasLSL−G12D+/− mice were infected with Cre-RFP resulting in loss of PTEN with activation of KRAS (PTENKO/Kras; Fig. 5A). These cells were combined with WT stroma and placed in the in vivo regeneration model (Fig. 5A). Within 6 weeks, combinations of PTENKO/Kras epithelium and WT stroma gave rise to endometrioid endometrial tumors that invaded into the renal parenchyma (Supplementary Fig. SSA and SSB). Mice harboring endometrial tumors were treated with progesterone or placebo (Fig. 5A). Unlike progesterone-sensitive PTENKO tumors, no response to hormonal therapy was observed in PTENKO/Kras tumors (Fig. 5B vs. Fig. 1B). These tumors persisted on the kidney, did not resolve despite coadministration of progesterone and estrogen and appeared similar to
placebo-treated controls (Fig. 5B, a and b vs. c and d). Histology (Fig. 5C, a vs. b and Supplementary Fig. S5C) and proliferation indices (Fig. 5D) were equivalent in progesterone- and placebo-treated PTENKO/Kras tumors. Loss of PTEN (Fig. 5C, c and d) and activation of the RAS pathway (Supplementary Fig. S5D) was confirmed in tumor epithelia. Obvious differences in the epithelial vs. stromal distribution of ER-α and PR (Fig. 5C, e–h) were not detected in progesterone- or placebo-treated cohorts. Importantly, almost all PTENKO/Kras tumor stroma appeared PR-negative in both hormonal conditions while abundant expression of epithelial PR was detected (Fig. 5C, g and h).

Activation of KRAS concomitant with cell autonomous loss of PTEN resulted in hormone-refractory endometrial cancers. Detection of KRAS and PTEN mutations are feasible with current clinical technologies. Given our findings, the status of both these genes should be tested in cohorts of patients with endometrial cancer as potential biomarkers for assessing response to progesterone therapy.

The expression of PR is diminished by methylation in the stroma of PTENKO/Kras progesterone-resistant tumors

Our data show that stromal PR signaling is essential in hormone-mediated resolution of PTENKO endometrial tumors (Fig. 4). Activation of KRAS in conjunction with PTEN-loss resulted in hormone-refractory cancers containing predominantly PR-negative stroma (Fig. 5C, g and h). We hypothesized that stromal PR protein levels would be decreased in PTENKO/Kras tumors as a result of epigenetic modification.

PR expression in the epithelium and stroma of PTENKO and PTENKO/Kras tumors was examined. On the basis of immunohistochemistry, much lower levels of PR were detected in the stroma but not epithelium of PTENKO/Kras compared with PTENKO tumors (Fig. 6A). Next, differences in epithelial and stromal PR protein and transcript were quantified in these carcinomas. Tumor stroma was color-marked via infection with GFP-expressing lentivirus.
Supplementary Fig. S6A), whereas tumor epithelia were marked with RFP during Cre lentiviral infection. PTENKO and PTENKO/Kras tumors were harvested, dissociated into single cells, and sorted by FACS (Supplementary Fig. S6A). Clear RFP (epithelial) and GFP (stromal) cellular populations could be visualized (Supplementary Fig. S6A). A Western blot analysis of isolated epithelia and stroma confirmed a significant decrease only in the stromal PR expression of PTENKO/Kras progesterone refractory tumors (Fig. 6B and Supplementary Fig. S6B).

Figure 5. Activation of Kras concomitant with PTEN-loss (PTENKO/Kras) caused progesterone resistance. A, schema for generation and hormonal therapy for PTENKO/Kras tumors. B, despite coadministration of estrogen and progesterone PTENKO/Kras tumors did not resolve (n = 8; a and b) and were similar to placebo-treated counterparts (n = 8; c and d). C, no obvious differences in histology were detected with progesterone treatment (a vs. b). PTEN was absent in tumor epithelia (c and d). The hormone receptor expression was similar between progesterone- and placebo-treated tumors (e and g vs. f and h). D, a high proliferation index was detected in tumors despite hormonal therapy (a vs. b). Scale bars are equal to 2 mm in B and 100 μm in C and D.
A potential mechanism for regulation of PR expression is epigenetic and through DNA methylation (42). Methylation of the PR gene (Pgr) has been reported in human endometrial cancer but was not specifically examined in stromal or epithelial compartments (43). Given these previous findings, we first examined PR transcript levels in the stroma of PTENKO/Kras compared with PTENKO tumors. There are two PR isoforms, PR-A and PR-B (44). PR-A is the dominant form of PR in the endometrium (45). Both transcripts overlap such that all nucleotides in PR-A are shared with PR-B (Supplementary Fig. S6C; ref. 44), consequently PR-A transcripts cannot be measured directly. Message levels of PR-A + PR-B or PR-B alone were determined in stroma and epithelia isolated from progesterone-sensitive and -refractory tumors (Fig. 6C and Supplementary Figure 6C).

Figure 6. Stromal PR is diminished in PTENKO/Kras progesterone-resistant tumors by methylation. A, abundant PR was detected in the epithelium and stroma of PTENKO tumors (a). Diminution in stromal PR was detected in PTENKO/Kras tumors (b). B, Western blot analysis confirmed a decrease in stromal PR of PTENKO/Kras tumors. Extracellular signal-regulated kinase (ERK) was a loading control. C, qPCR revealed a significant decrease in PR-A + PR-B transcript in tumor stroma of PTENKO/Kras compared with PTENKO tumors (a). A nonsignificant decrease in levels of PR-B transcript was detected (b). D, schematic of the PR-A promoter with CpG islands is shown. On the basis of bisulfite sequencing, a higher percentage of the PR-A promoter CpG islands was methylated in tumor stroma of PTENKO/Kras compared with PTENKO tumors. Three independent tumors for each group were analyzed. Scale bars, 50 μm.
Fig. S6C). Decreased transcript levels of PR-A+PR-B were detected in stroma but not the epithelium of PTENKO/Kras tumors compared with their hormone-sensitive PTENKO counterparts (Fig. 6C, a and Supplementary Fig. S6C). Decreased PR-B transcript levels were also observed in PTENKO/Kras tumor stroma, but this was not statistically significant (Fig. 6C, b). Results suggest that transcriptional modulation of PR primarily occurred through repression of PR-A. Methylhation-specific PCR was conducted to examine DNA methylation in the PR-A promoter (Supplementary Fig. S6D). A greater proportion of methylated PR-A DNA was detected in the stroma of PTENKO/Kras tumors (Supplementary Fig. S6e). Bisulfite sequencing confirmed methylation of DNA at the PR-A promoter. Minimal methylation of the PR-A promoter was detected in PTENKO tumor stroma, whereas the majority of PR-A promoter Cpg islands were methylated in the stroma of PTENKO/Kras tumors (Fig. 6D). These findings confirm that DNA methylation of PR-A transcription and expression in the stroma of progesterone refractory PTENKO/Kras tumors.

In this model, activation of KRAS with PTEN-loss in tumor epithelium may lead to the secretion of paracrine factors that modulate the epigenetic landscape of surrounding tumor stroma. This signaling cascade from epithelium to stroma could trigger a series of events ultimately resulting in methylation of the PR gene. Methylation of PR in hormone-refractory endometrial tumors may be among one of the mechanisms causing repression of PR transcription and expression in tumor stroma.

Overexpression of exogenous PR in tumor stroma sensitized PTENKO/Kras tumors to hormonal therapy

Progesterone-resistant PTENKO/Kras tumors had decreased expression of stromal PR due to silencing of the PR gene in the tumor microenvironment. We hypothesized that loss of stromal PR is a key mechanism leading to hormone resistance in endometrial tumors. If this hypothesis is correct, reexpression of PR in the tumor stroma would be sufficient to sensitize PTENKO/Kras tumors to hormonal therapy.

To address this question, the human PR (hPR) was cloned into a GFP-expressing lentiviral vector (Supplementary Fig. S7Aa). WT neonatal stroma was infected with hPR-GFP or GFP control lentivirus (Fig. 7A). Overexpression of PR-A and PR-B was confirmed (Supplementary Fig. S7Ab and S7Ac). Stroma expressing GFP or hPR-GFP was FACS isolated, combined with Cre-RFP–infected Pten<sup>loxP/loxPKras<sup>LSL-G12D/+</sup></sup> epithelia and reggenerated in vivo (Fig. 7A). This resulted in activation of KRAS and PTEN-loss in tumor epithelium with or without overexpression of human PR in the stroma. After 6 weeks, tumor histology was similar in pretherapy PTENKO/Kras cancers regardless of PR overexpression in the stroma (Supplementary Fig. S7B). Mice were treated with estrogen plus progesterone cotherapy (progesterone) or estrogen plus placebo (placebo; Fig. 7A). Tumors with WT GFP stroma were large and persisted in both progesterone- and placebo-treated cohorts (Fig. 7B, a and b vs. d and e). Stromal PR was significantly diminished in these tumors (Fig. 7B, c and f). In contrast, when exogenous hPR was expressed in tumor stroma the majority of PTENKO/Kras tumor tissue resolved with progesterone administration (Fig. 7B, g and h and Supplementary Fig. S7Da and S7Db). This effect was mediated by progesterone signaling as placebo-treated tumors expressing stromal hPR persisted (Fig. 7B, j and k and Supplementary Fig. S7Dc and S7Dd). Unlike PTENKO/Kras with WT-GFP stroma, tumors with hPR-GFP stroma had abundant expression of PR in the tumor microenvironment (Fig. 7B, c and f vs. i and l). Deletion of PTEN and RAS pathway activation was confirmed in tumor epithelia (Supplementary Fig. S7C).

In PTENKO tumors, we showed that expression of stromal PR was essential for progesterone-mediated resolution of the carcinoma (Fig. 4). Here, we show that reexpression of PR in the tumor stroma is sufficient to sensitize the hormone-refractory PTENKO/Kras endometrial tumors to progesterone therapy (Fig. 7). Collectively, our findings show that signaling through stromal PR is sufficient and necessary for mediating antitumor effects of progesterone therapy in endometrial cancer.

Discussion

The prevailing dogma in clinical practice assumes that progesterone exerts its therapeutic effects through tumor epithelia, which are the more abundant cell type in endometrial carcinomas. Studies have attempted to assess PR expression as a predicative biomarker for response to progesterone therapy with limited success (37, 38). Here, we show that progesterone hormonal therapy works by paracrine signaling through PR in the endometrial tumor microenvironment. Loss of stromal PR in PTENKO hormone-sensitive tumors induced progesterone resistance. Activation of KRAS concomitant with PTEN-loss initiated progesterone-resistant endometrial tumors that had measurable loss of stromal PR. Add-back of exogenous PR in the stroma of hormone-refractory tumors was sufficient to induce sensitivity to progesterone therapy. For the first time, we show that signaling through the stromal PR axis is sufficient and necessary for successful resolution of endometrial tumors with progesterone.

Cross-talk between epithelium and stroma is essential for neoplastic transformation in many hormonally regulated tissues (18, 20, 46, 47). One way this cross-talk occurs is through reciprocal signals regulating hormone receptor expression or activity in each cellular compartment. Previously, we showed that cancer-initiating fibroblast growth factor signals originating from stroma increased androgen receptor levels in prostate tumor epithelia, resulting in androgen-independent growth (20). Here, we show that oncogenic insults in endometrial tumor epithelia decreased levels of PR in tumor stroma inducing <i>de novo</i> progesterone resistance. Epigenetic modification of the tumor microenvironment has emerged as a potential regulator of tumor initiation and propagation in breast (48) and prostate (18) cancers. Our results show that epigenetic regulation of tumor stroma can induce drug resistance. Defining mechanisms and site of origin for innate or acquired resistance to hormonal therapy in human endometrial cancer trials will have immense translational application.

We find that endometrial tumors resulting solely from epithelial PTEN-loss are highly sensitive to progesterone anti-tumor effects. These results contradict findings from a <i>Pten<sup>−/−</sup></i>...
transgenic model (29), but there are two major differences in the studies. In our model, Pten is deleted only in tumor epithelia emulating the epithelial-specific loss of PTEN seen in human endometrial cancers. Conversely, in Pten<sup>1/-</sup> transgenic mice Pten is deleted in tumor epithelia and stroma. In both models, mice are oophorectomized and treated with progesterone. But
in our study, estrogen is coadministered with progesterone resulting in successful hormonal therapy for PTEN-null tumors. The discrepancy in progesterone sensitivity in these two models could be explained by stromal *Pten* status and the absence or presence of estrogen during treatment.

Although loss of PTEN function is the most common genetic change in endometrial cancer, in 21% of cases it occurs in conjunction with KRAS activation (23). Despite the prevalence of this coexisting genetic alteration, previous studies have not assessed the sensitivity of endometrial tumors resulting from PTEN-loss and KRAS activation to hormonal therapy. When epithelial activation of KRAS was coupled with PTEN-loss, stromal PR expression was drastically reduced resulting in progesterone resistance. In another *Pten/Kras* transgenic endometrial cancer model (30), decreased expression of PR was reported. However, this study did not assess sensitivity of these tumors to progesterone or examine PR distribution in tumor epithelium or stroma. The lack of response to progesterone therapy in PTENKO/Kras tumors in our model may explain why many PTEN-null endometrial cancers in clinical series are refractory to progesterone therapy (27, 28). Coexistence of other genetic changes, such as activation of KRAS, in these tumors may induce progesterone resistance. In a mouse pancreatic tumor model, epithelial-specific activation of KRAS could alter the tumor microenvironment by non–cell autonomous mechanisms (49). Similarly, we suspect that activation of KRAS in PTEN-null tumor epithelia results in secretion of paracrine factors that can modulate the endometrial tumor microenvironment ultimately silencing PR expression.

This study reveals three imminently testable biomarkers for progesterone sensitivity to be assessed in future clinical trials. These biomarkers include *PTEN* and *KRAS* status in the tumor epithelia and expression of PR in tumor stroma. Molecular analysis of tumors with reliable biomarkers of response to hormonal therapy can individualize treatment options for the 49,000 U.S. women diagnosed with endometrial cancer annually (4). Stromal loss of PR through epigenetic silencing was a key mechanism inducing progesterone resistance in our model. Importantly, stromal reexpression of PR in hormone-refractory cancers sensitized these tumors to progesterone therapeutic effects. This finding may have critical clinical implications as it shows that modulation of the tumor microenvironment can reverse hormone resistance in endometrial tumors. In future work, we will test if stromal-specific delivery of DNA methyltransferase inhibitors may be an effective way to reestablish hormone-refractory endometrial cancers to progesterone therapy.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.M. Janzen, S. Memarzadeh

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Progestosterone Receptor Signaling in the Microenvironment of Endometrial Cancer Influences Its Response to Hormonal Therapy

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