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Progesterone receptor signaling in the microenvironment of endometrial cancer influences its response to hormonal therapy

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Abstract

Progesterone, an agonist for the progesterone receptor (PR), can be an efficacious and well-tolerated treatment in endometrial cancer. The clinical use of progesterone is limited due to the lack of biomarkers that predict hormone sensitivity. Despite its efficacy in cancer therapy, mechanisms and site of action for progesterone remain unknown. Using an *in vivo* endometrial cancer mouse model driven by clinically relevant genetic changes but dichotomous responses to hormonal therapy, we demonstrate that signaling through stromal PR is necessary and sufficient for progesterone anti-tumor 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 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 demonstrate that signaling through stromal PR is sufficient for inducing hormone responsiveness. Our findings suggest that epigenetic de-repression of stromal *PR* could be a potential therapeutic target for sensitizing hormone refractory endometrial tumors to progesterone therapy. Based on these results, stromal expression of PR may emerge as a reliable biomarker in predicting response to hormonal therapy.

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Introduction

Typically, antagonists of steroid receptors are exploited in the therapy of hormonally regulated carcinomas (1-3). Endometrial cancer is unique in that agonistic actions of progesterone exert anti-tumor effects. Uterine cancers are the most prevalent gynecologic malignancy in the western world with a rising incidence in the U.S. (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 progesterone. Activation of oncogenes or loss of tumor suppressors initiate 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 of 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 progesterone causes atrophy of the normal endometrium, it is administered clinically as a single agent in the therapy of endometrial cancer. Despite five decades of clinical use, the anti-tumor mechanisms and site of action for progesterone therapy remain unknown. Molecular mechanisms underlying progesterone resistance or sensitivity are also poorly understood.

Progesterone is well tolerated, easily administered and has minimal side effects. Subsets of patients respond to progesterone while others have progression of disease while on hormonal therapy (11). Response rates to progesterone range from 11-50% 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 of endometrial cancer involves removal of reproductive organs, at times coupled with adjuvant radiation or chemotherapy (13). While 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 endometrial cancer patients.

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 utilized our dual compartment regeneration system as an in vivo pre-clinical 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, while 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 demonstrate that signaling through stromal progesterone receptor is necessary and sufficient for anti-tumor effects of progesterone therapy.

Material and Methods

Animals

WT C57BL/6, *Pten*^{loxP/loxP} (C;129S4-*Pten*^{tm1Hwu/J}), *Kras*^{LSL-G12D/+} (B6.129S4-*Kras*^{tm4Tyj/J}) and CB17Scid/Scid mice were from Jackson Laboratory. *PR*^{loxP/loxP} (PRCE) mice were from Dr. Luisa Iruela-Arispe. *Pten*^{loxP/loxP} *Kras*^{LSL-G12D/+} mice were generated by crossing the *Kras*^{LSL-G12D} allele into the *Pten*^{loxP/loxP} background. Mice were maintained in accordance with University of California Los Angeles (UCLA), 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), 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 performed 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 FACS sorting as reported (21). To obtain adult endometrial epithelia, mouse uteri were dissected, cut into fragments and incubated in 1% trypsin for 45min at 4°C. Luminal epithelia were separated from underlying stroma with a fine forceps and digested with 0.8 mg/ml collagenase in DMEM/10% FBS with 5 µg/ml insulin and 0.5 mg/ml DNase for 1.5h 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 min for fluorescent activator cell sorting (FACS). Endometrial epithelia 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 performed 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, re-suspended in collagen (BDBiosciences; 354236) and dispensed into grafts. Endometrial grafts were implanted under the kidney capsule of oophorectomized CB17Scid/Scid mice and regenerated for 6-8 weeks with an estrogen pellet (60-d 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 eight week course of progesterone therapy was administered by subcutaneous implantation of a time release pellet (60-d 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-d time release, 100 mg progesterone placebo/pellet; SC-111 Innovative Research of America). Estrogen pellets (60-d 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 performed under the UCLA DLAM regulations. Serum hormone levels in mice were verified using estradiol EIA and progesterone EIA kits (Cayman Chemical).

Immunohistochemistry

Immunohistochemistry was performed as previously described (17) using antibodies listed in Supplementary Tables. To quantify expression of Ki67, TUNEL and cleaved caspase-3, 5-10 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 μ g/ml insulin and 0.5 mg/ml DNase for 2 hr 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 was performed 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

Western blotting of isolated cell fractions was performed 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 bisulfate 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 \pm SD. To determine significance, comparisons were performed using a two-tailed t test for groups of two or one-way ANOVA with Tukey honestly significant difference criterion 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) while 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 sub-renal space of oophorectomized mice supplemented with estrogen via implantation of an 8 week time-release 17 β -estradiol pellet (17) (Fig. 1A). 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&B). Given that endometrial cancer commonly occurs in patients with high estrogen levels (32), tumor bearing mice were re-implanted with new estradiol pellets to maintain a hyper-estrogenic state. Simultaneously half of these mice were implanted with progesterone pellets while 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&b), while only small cysts were found on kidneys of progesterone treated animals (Fig. 1B c&d). The histology of placebo treated PTENKO tumors was endometrioid with epithelial-specific loss of PTEN (Fig. 1C a&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&f and Supplementary Fig. S1D). Abundant expression of epithelial and stromal estrogen receptor α (Esr1 or ER α), and progesterone receptor (Pgr or PR) was detected in both progesterone and placebo treated tissue (Fig. 1C c,d,g&h). Progesterone therapy significantly diminished proliferation of PTEN-null epithelium as measured by Ki67 (Mki67) expression (Fig. 1D).

Our findings demonstrate that endometrial tumors resulting from epithelial loss of PTEN are exquisitely sensitive to progesterone hormonal therapy. Observations in this mouse model suggest that loss of *Pten* as a single genetic change in the tumor epithelia should be investigated as a potential biomarker of response to progesterone therapy in patients with endometrial cancer.

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 with a few weeks of therapy, while others require more than six months of progesterone administration to demonstrate 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), while 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,l vs. a,f,k) coupled with a significant decrease in proliferation (Fig. 2C and Supplementary Fig. S2A b vs. a). Progressive tumor clearance was detected at 4 (Fig. 2B c,h,m) and 6 weeks (Fig. 2B d,i,n). Although the tumor proliferation plateaued between 2 and 6 weeks (Fig. 2C), apoptotic cell death rose

significantly during this time period (Supplementary Fig. S2B b-d and g-i). Complete tumor resolution was observed at 8 weeks (Fig. 2B e,j,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 while in the second phase cell death ensued, resulting in resolution of the remaining tumor cells. Findings here demonstrate that progesterone anti-tumor effects in PTENKO tumors are quick but kinetic resulting from a combination of decreased proliferation and increased cell death.

Co-administration of estrogen was essential for progesterone mediated anti-tumor effects in PTEN null endometrial tumors

While 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 of 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 co-therapy) (Fig. 3A). Prior to initiating treatment, establishment of pre-therapy tumors was confirmed (Fig. 3B a-c). Co-therapy with estrogen and progesterone resolved PTENKO endometrial tumors demonstrated by development of cysts (Fig. 3B e-g) with decreased epithelial proliferation compared to pre-therapy tumors (Supplementary Fig. S3A). Surprisingly, PTENKO tumors treated with progesterone alone persisted (Fig. 3B i-k) and had a proliferation index comparable to pre-therapy 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. 3 Cc) compared to estrogen and progesterone co-treated (Fig. 3 Cb) and pre-therapy counterparts (Fig. 3 Ca). Abundant expression of epithelial PR was detected in all conditions (Fig. 3B d,h,l and Fig. 3C).

Findings here demonstrate that co-administration of estrogen with progesterone is required in hormonal therapy of 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 endogenous 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 endometrial cancer patients may account for the wide variation in time required to achieve therapeutic responses (33).

Stromal deletion of progesterone receptor 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 progesterone receptor (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. 3B1 and Fig. 3Cc). 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 *PR^{loxP/loxP}* (39) endometrial stromal cells were infected with Cre-expressing or control lentivirus marked with green fluorescent protein (GFP) and cultured short-term (Fig. 4A). Expression of Cre in these cells resulted in loss of PR-A and PR-B (Supplementary Fig. S4A). Combinations of FACS isolated *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 & 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&b) while tumors persisted in placebo treated controls (Fig. 4B d&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&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&h vs. j&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&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 demonstrate that signaling through stromal PR is essential for mediating anti-tumor 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 demonstrate that signaling through stromal PR decreases epithelial tumor proliferation and induces apoptotic cell death in endometrial tumor tissue. We suspect that mediators of the anti-tumor 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 co-existing 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/loxP} Kras^{LSL-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 six weeks, combinations of PTENKO/Kras epithelium and WT stroma gave rise to endometrioid endometrial tumors that invaded into the renal parenchyma (Supplementary Fig. S5A&B). 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 co-administration of progesterone and estrogen and appeared similar to placebo treated controls (Fig. 5B a&b vs. c&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&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&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 endometrial cancer patients as potential biomarkers for assessing response to progesterone therapy.

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

Our data demonstrates 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&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. Based on immunohistochemistry, much lower levels of PR were detected in the stroma but not epithelia of PTENKO/Kras compared to 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) while 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 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).

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 to 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* (44) (Supplementary Fig. S6C), 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 Fig. S6C). Decreased transcript levels of *PR-A+PR-B* were detected in stroma but not the epithelium of PTENKO/Kras tumors compared to their hormone sensitive PTENKO counterparts (Fig. 6Ca and Supplementary Fig. S6C). Decreased *PR-B* transcript levels were also observed in PTENKO/Kras tumor stroma, but this was not statistically significant (Fig. 6Cb). Results suggest that transcriptional modulation of *PR* primarily occurred through repression of *PR-A*. Methylation specific PCR was performed 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, while 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* decreases transcription and thus expression of PR 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.

Over-expression 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, re-expression of PR in the tumor stroma would be sufficient to sensitize PTENKO/Kras tumors to hormonal therapy.

To address this question the human progesterone receptor (hPR) was cloned into a GFP expressing lentiviral vector (Supplementary Fig. S7Aa). Wild type neonatal stroma was infected with hPR-GFP or GFP control lentivirus (Fig. 7A). Over-expression of PR-A and PR-B was confirmed (Supplementary Fig. S7A b&c). Stroma expressing GFP or hPR-GFP was FACS isolated, combined with Cre-RFP infected *Pten^{loxP/loxP} Kras^{LSL-G12D/+}* epithelia and regenerated in vivo (Fig. 7A). This resulted in activation of KRAS and PTEN-loss in tumor epithelium with or without over-expression of human PR in the stroma. After 6 weeks, tumor histology was similar in pre-therapy PTENKO/Kras cancers regardless of PR over-expression in the stroma (Fig. S7B). Mice were treated with estrogen plus progesterone co-therapy (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&b vs. d&e). Stromal PR was significantly diminished in these tumors (Fig. 7B c&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&h and Supplementary Fig. S7D a&b). This effect was mediated by progesterone signaling as placebo treated tumors expressing stromal hPR persisted (Fig. 7B j&k and Supplementary Fig. S7D c&d). Unlike PTENKO/Kras with WT-GFP stroma, tumors with hPR-GFP stroma had abundant expression of PR in the tumor microenvironment (Fig. 7B c&f vs. i&l). Deletion of PTEN and RAS pathway activation was confirmed in tumor epithelia (Supplementary Fig. S7C).

In PTENKO tumors we demonstrated that expression of stromal PR was essential for progesterone mediated resolution of the carcinoma (Fig. 4). Here we demonstrate that re-expression 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 demonstrate that signaling through stromal PR is sufficient and necessary for mediating anti-tumor 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 demonstrate 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 demonstrate 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 demonstrated 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 de novo 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 *Pten*^{+/-} 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*^{+/-} 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 co-administered with progesterone resulting in successful hormonal therapy of 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 co-existing 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 progesterone receptor 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). Co-existence 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 re-expression of PR in hormone refractory cancers sensitized these tumors to progesterone therapeutic effects. This finding may have critical clinical implications as it demonstrates 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 re-sensitize hormone refractory endometrial cancers to progesterone therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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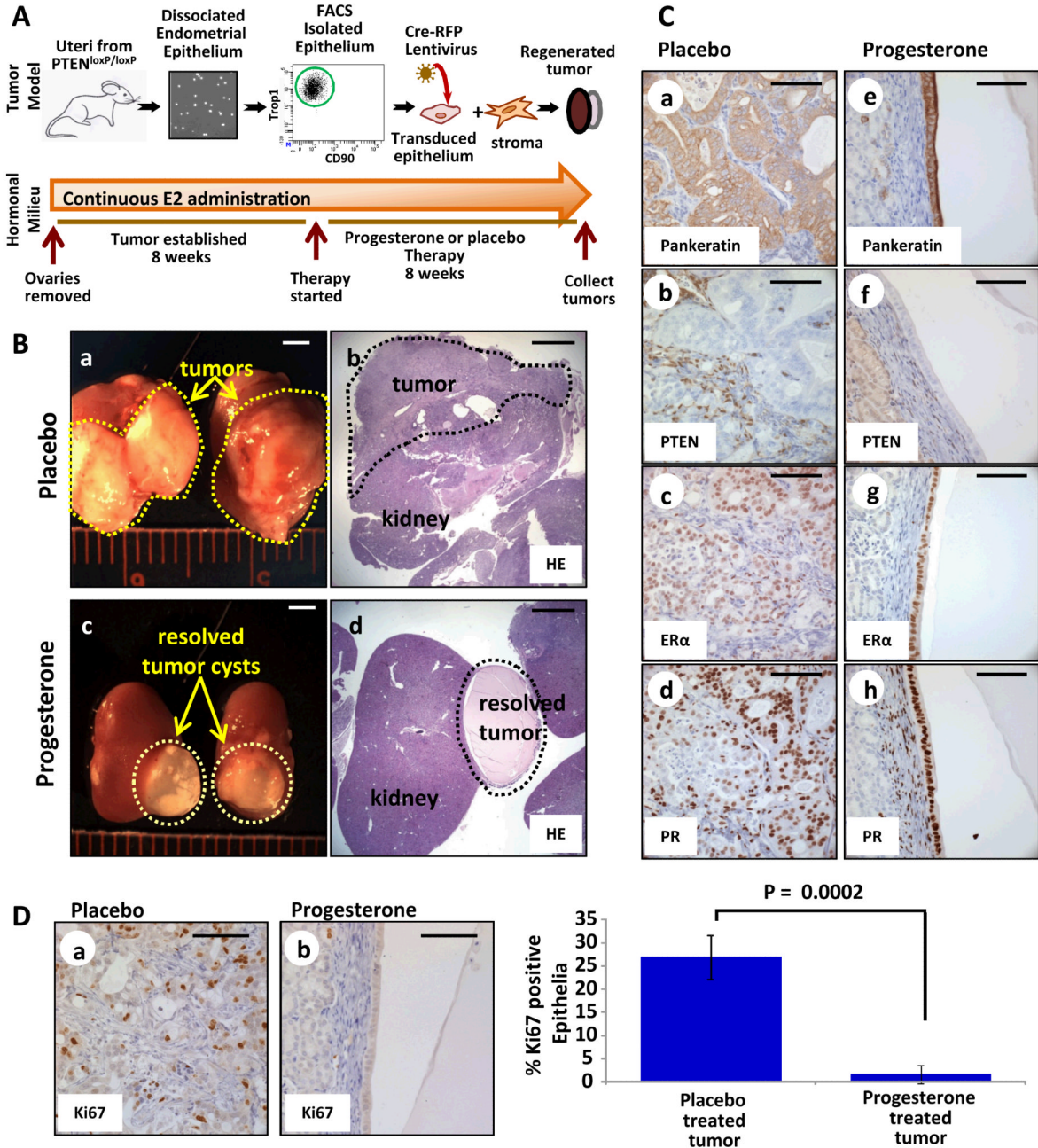


Figure 1. Progesterone therapy resulted in resolution of endometrial tumors initiated by epithelial loss of PTEN (PTENKO)
(A) Schema for in vivo therapy. **(B)** Large tumors were found in placebo treated mice (n=8) (a&b). Resolution of tumors was noted with progesterone therapy (n=8) (c&d). **(C)** Residual cysts in progesterone treated mice contained a single epithelial layer compared to persistent tumor epithelia in placebo treated controls (e vs. a). PTEN was absent in all epithelia (b&f). Epithelial and stromal ER α (c&g) and PR (d&h) were detected with progesterone or placebo. **(D)** A decrease in proliferating epithelia was detected upon progesterone administration (a vs. b). Scale bars equal 2 mm in B, and 100 μ m in C&D.

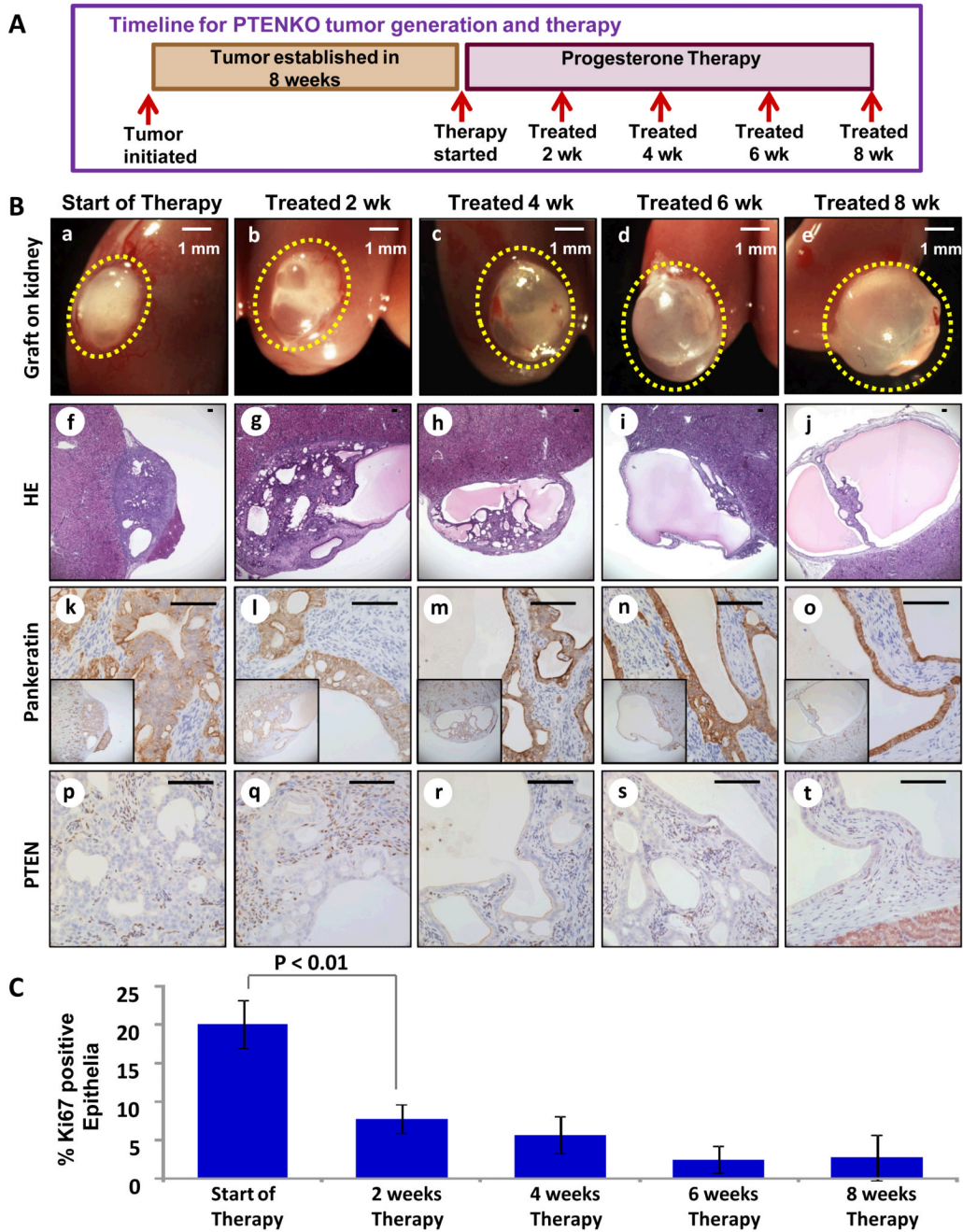


Figure 2. Resolution of PTENKO tumors is gradual but complete within 8 weeks

(A) Established PTENKO tumors were treated with estrogen and progesterone for 2, 4, 6 or 8 weeks (n=4 at each time point). (B) Tumor clearing was detected over time (a-e). Histologic analysis confirmed resolution of tumor mass (f-j). The histology of epithelia marked by pankeratin demonstrated resolution of tumor epithelia into normal appearing glands (k-o). Epithelial loss of PTEN was confirmed (p-t). (C) A progressive decrease in the proliferation of tumor epithelia was observed throughout therapy. Scale bars are 100 μ m except where noted.

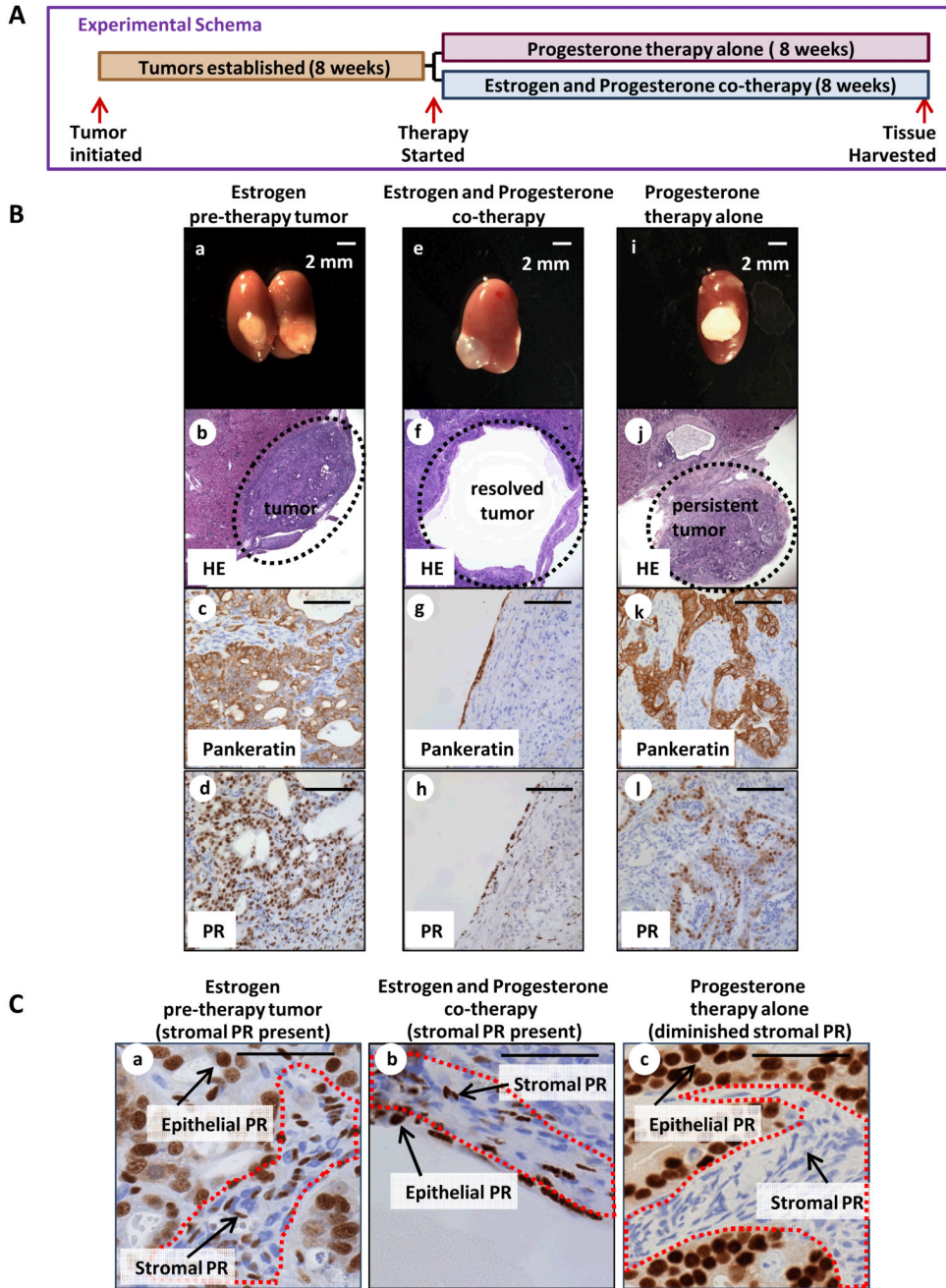


Figure 3. Effective therapy with progesterone requires co-administration of estrogen
(A) Established PTENKO tumors were treated with: (1) estrogen with progesterone co-therapy (n=4) or (2) progesterone therapy alone (n=8). **(B)** In the absence of estrogen, progesterone hormonal therapy failed to resolve PTENKO tumors. Establishment of tumor was confirmed prior to therapy (a-d). Resolution of tumors was observed with estrogen and progesterone co-therapy (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&b). Scale bars equal 100 μ m except where noted.

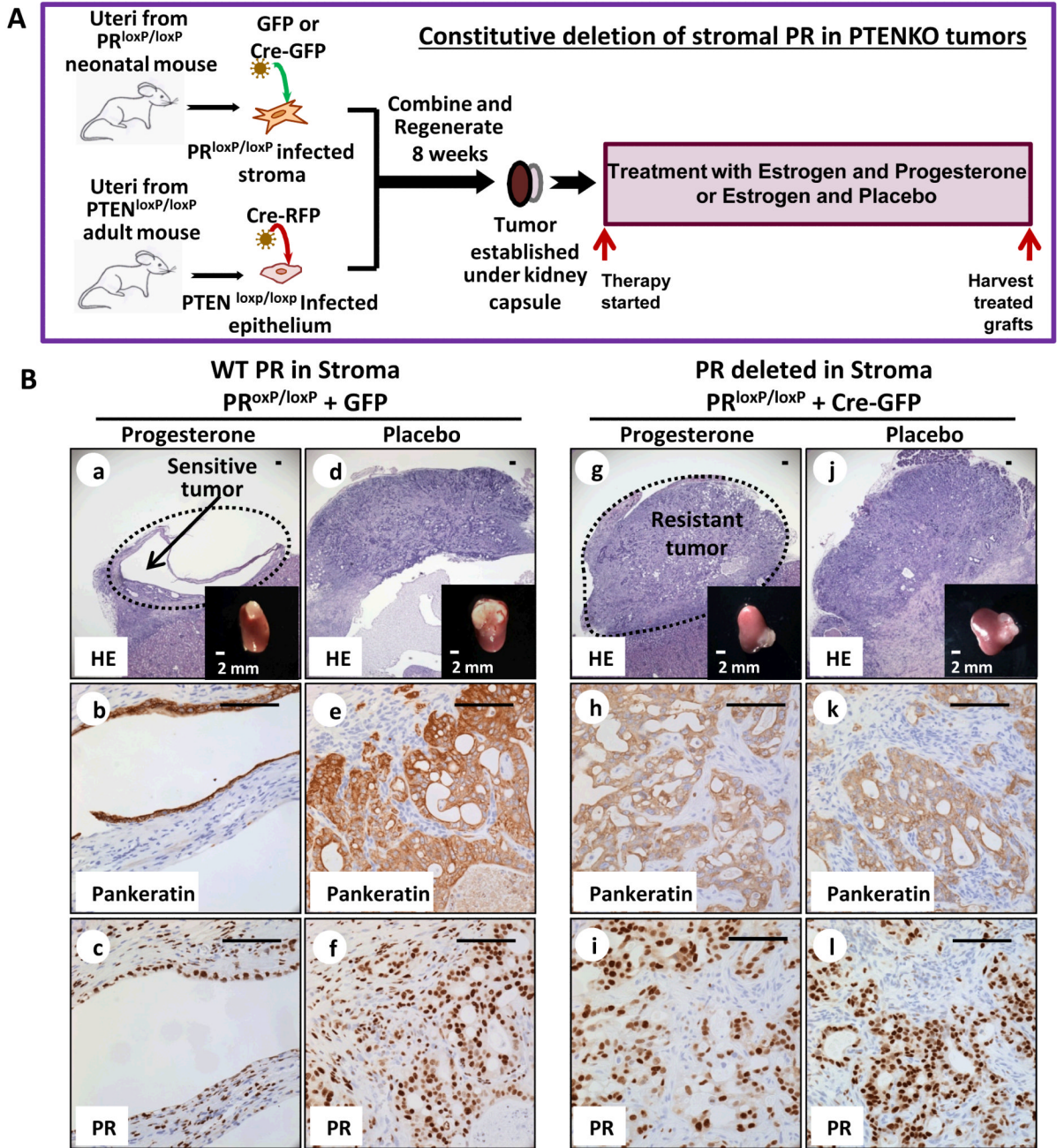


Figure 4. Progesterone therapeutic effects are mediated through the stromal progesterone receptor

(A) Strategy for deletion of *PR* in the stroma of PTENKO tumors. (B) Stromal loss of *PR* induced progesterone resistance. PTEN null endometrial tumors with stromal *PR* expression regressed with progesterone therapy (n=6) (a-c) but persisted in the placebo treated group (n=6) (d-f). Conversely, when stromal *PR* was deleted, PTENKO endometrial tumors did not respond to progesterone (n=8) (g-i) and the histology was similar to placebo treated controls (n=8) (j-l). Cre-GFP induced loss of stromal *PR* was confirmed (i&l vs. c&f). Scale bars are 100 μ m except where noted.

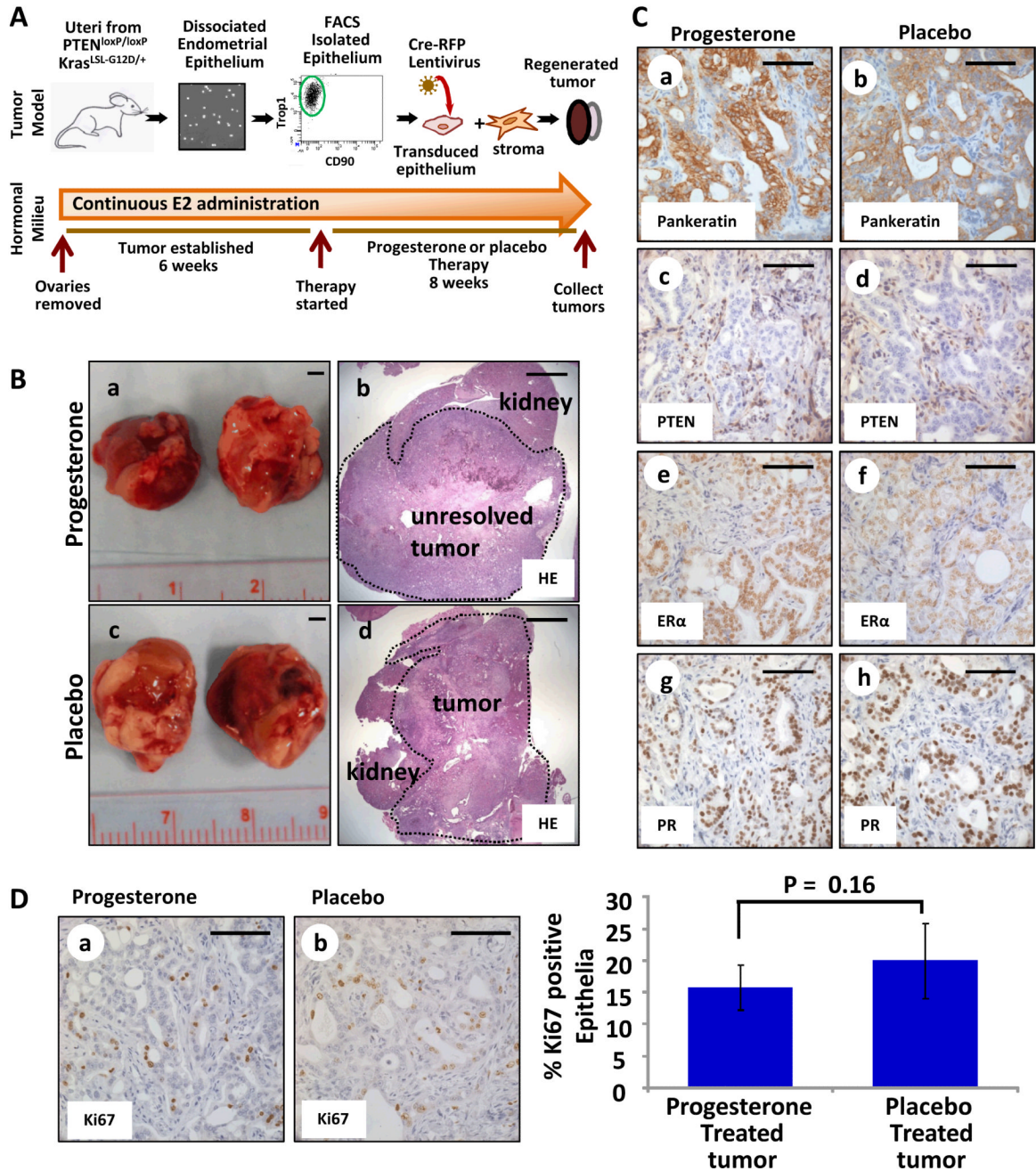


Figure 5. Activation of Kras concomitant with PTEN loss (PTENKO/Kras) caused progesterone resistance

(A) Schema for generation and hormonal therapy of PTENKO/Kras tumors (B) Despite co-administration of estrogen and progesterone PTENKO/Kras tumors did not resolve (n=8) (a&b) and were similar to placebo treated counterparts (n=8) (c&d). (C) No obvious differences in histology was detected with progesterone treatment (a vs. b). PTEN was absent in tumor epithelia (c&d). The hormone receptor expression was similar between progesterone and placebo treated tumors (e&g vs. f&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&D.

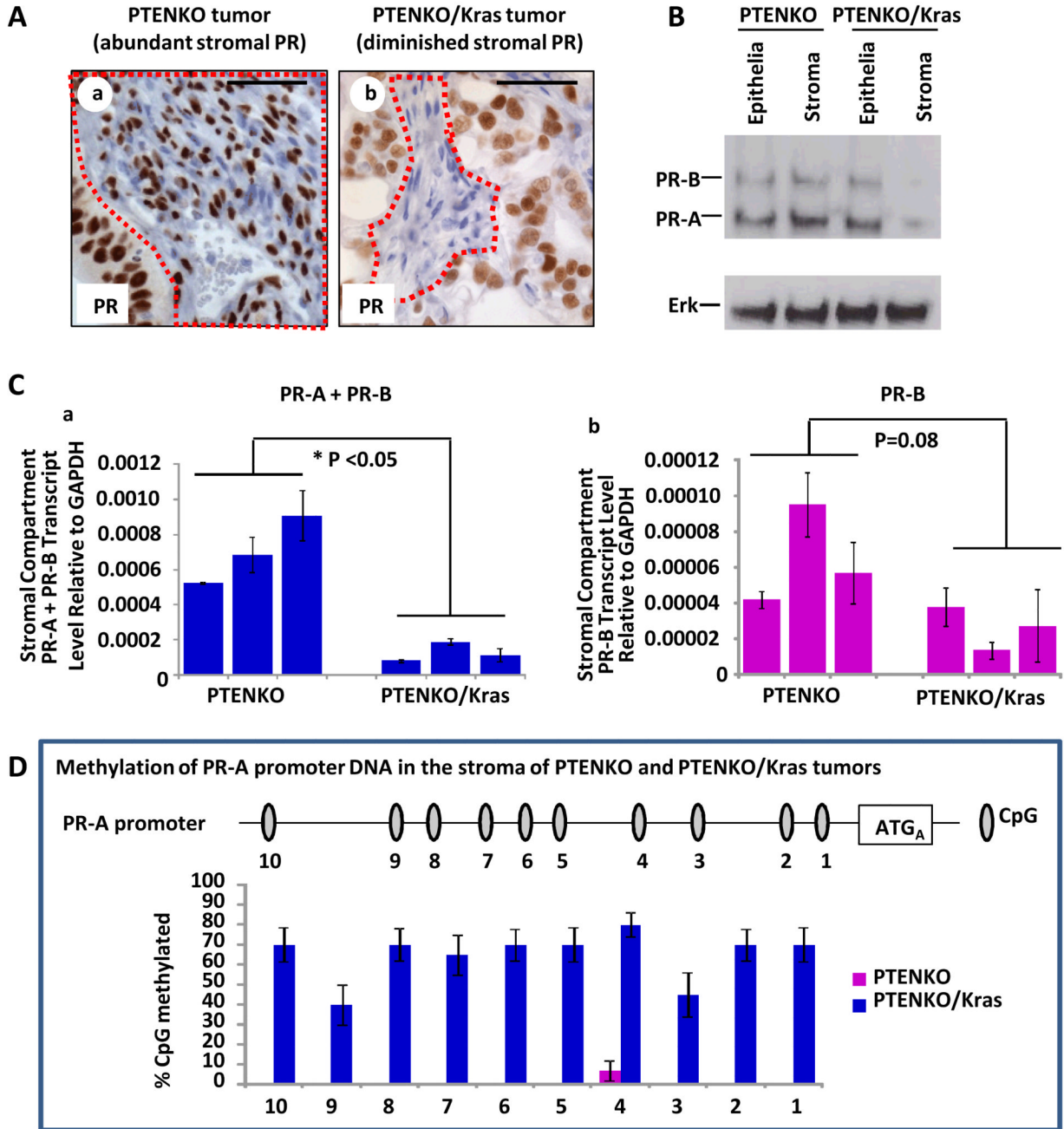


Figure 6. Stromal PR is diminished in PTENKO/Kras progesterone resistant tumors by methylation

(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 confirmed a decrease in stromal PR of PTENKO/Kras tumors. ERK was a loading control. (C) Q-PCR revealed a significant decrease in *PR-A*+ *PR-B* transcript in tumor stroma of PTENKO/Kras compared to PTENKO tumors (a). A non-significant decrease in levels of *PR-B* transcript was detected (b). (D) Schematic of the *PR-A* promoter with CpG islands is shown. Based on bisulfite sequencing, a higher percentage of the *PR-A* promoter CpG

islands were methylated in tumor stroma of PTENKO/Kras compared to PTENKO tumors. Three independent tumors for each group were analyzed. Scale bars equal 50 μm .

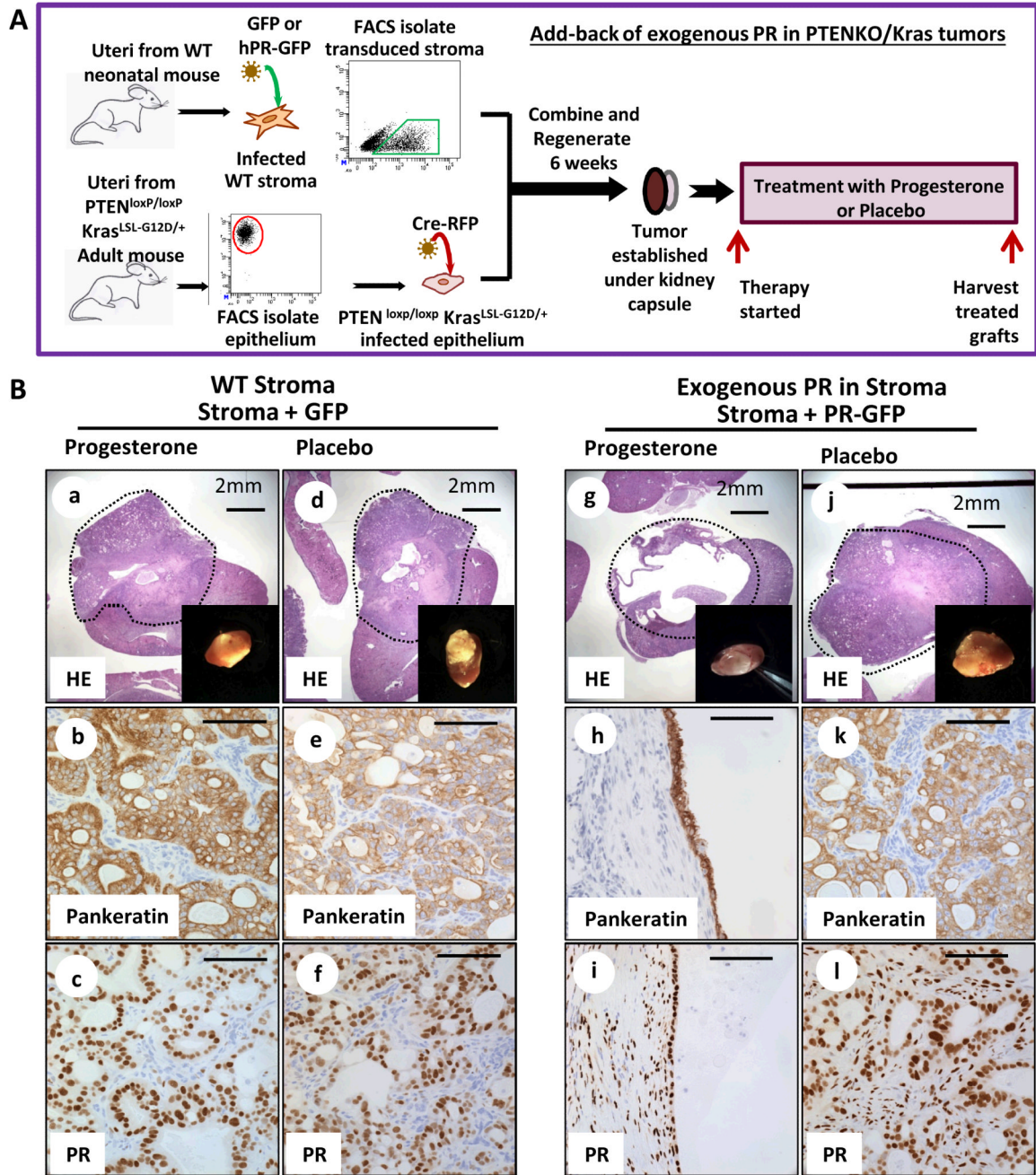


Figure 7. Expression of exogenous progesterone receptor in the stroma of PTENKO/Kras tumors shifted their biologic behavior from hormone refractory to hormone sensitive cancers (A) Experimental strategy for over-expression of PR in the stroma of PTENKO/Kras tumors. (B) Over expression of human PR in the stroma of PTENKO/Kras tumors facilitated tumor resolution in response to progesterone therapy. PTENKO/Kras tumors expressing GFP in the stroma were resistant to progesterone therapy (n=2) (a-c) and resembled placebo treated counterparts (n=2) (d-f). Tumors over-expressing stromal PR responded to progesterone (n=3) evidenced by formation of cysts attached to the kidney (g-i) compared to placebo (j-l) treated controls (n=3). Scale bars equal 100µm except where noted.