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Progesterone receptor membrane component 1 deficiency attenuates growth while promoting chemosensitivity of human endometrial xenograft tumors

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Abstract

Endometrial cancer is the leading gynecologic cancer in women in the United States with 52,630 women predicted to be diagnosed with the disease in 2014. The objective of this study was to determine if progesterone (P4) receptor membrane component 1 (PGRMC1) influenced endometrial cancer cell viability in response to chemotherapy *in vitro* and *in vivo*. A Jentiviral-based shRNA knockdown approach was used to generate stable PGRMC1-intact and PGRMC1-deplete Ishikawa endometrial cancer cell lines that also lacked expression of the classical progesterone receptor (PGR). Progesterone treatment inhibited mitosis of PGRMC1-intact, but not PGRMC1-deplete cells, suggesting that PGRMC1 mediates the anti-mitotic actions of P4. To test the hypothesis that PGRMC1 attenuates chemotherapy-induced apoptosis, PGRMC1-intact and PGRMC1-deplete cells were treated *in vitro* with vehicle, P4 (1 μ M), doxorubicin (Dox. 2 μ g/ml), or P4 + Dox for 48 h. Doxorubicin treatment of PGRMC1-intact cells resulted in a significant increase in cell death; however, co-treatment with P4 significantly attenuated Dex-induced cell death. This response to P₄ was lost in PGRMC1-deplete cells. To extend these observations *in vivo*, a xenograft model was employed where PGRMC1-intact and PGRMC1-deplete endometrial tumors were generated following subcutaneous and intraperitonea l inoculation of immunocompromised NOD/SCIO and nude mice, respectively. Tumors derived from PGRMC1-deplete cells grew slower than tumors from PGRMC1-intact cells. Mice harboring endometrial

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Conflict of interest

JJ Peluso was awarded a patent on the non-genomic regulators of P4 action. The remaining authors have no conflicts of interest to disclose.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2014.09.036.

tumors were then given three treatments of vehicle (1:1 cremophor EL: ethanol + 0.9% saline) or chemotherapy [Paclitaxel (15 mg/kg, i.p.) followed after an interval of 30 minutes by CARBOplatin (50 mg/kg)] at five day intervals. In response to chemotherapy, tumor volume decreased approximately four-fold more in PGRMC1-deplete tumors when compared with PGRMC1 intact control tumors, suggesting that PGRMC1 promotes tumor cell viability during chemotherapeutic stress. In sum, these *in vitro* and *in vivo* findings demonstrate that PGRMC1 plays a prominent role in the growth and chemoresistance of human endometrial tumors.

Keywords

Cancer; Chemotherapy; Endometrial; PGRMC1; Progesterone; Xenograft

Introduction

Endometrial cancer is the most common gynecologic malignancy, and its incidence in the United States is predicted to reach 52,630 in 2014 with 8590 succumbing to the disease [1]. Fortunately, surgical intervention in patients diagnosed with stage 1 or 2 lowgrade endometrioid endometrial cancer is highly effective as evidenced by the overall survival rate of 95% at five years. In contrast, high-grade endometrial cancers diagnosed at late stage of progression have a much poorer prognosis and an increased incidence of recurrence. Endometrial endometrioid cancer is most common among postmenopausal women; yet, 5% of women are diagnosed with the disease prior to age 40. The most routine treatment for endometrial cancer is total hysterectomy followed by radiation therapy [2,3]. For women under 40 years of age, a uterine sparing non-surgical approach is often employed with low-grade endometrial cancer confined to the uterus. This is especially true for women who wish to maintain reproductive options.

Progestogen therapy is an effective non-toxic temporizing fertilitysparing alternative to a surgical intervention [2-4]. Progestogens are known to attenuate estrogen-induced endometrial epithelial cell proliferation while promoting differentiation of both epithelial and stromal cells. Although the response rate to progestogen therapy approaches 60% in premenopausal women with well-differentiated endometrial cancer [4,5], it is only effective in 10-15% of women with advanced or recurrent endometrial cancer [6,7]. As such, patient age, tumor histophenotype and grade greatly determine the efficacy of this approach.

Low-grade endometrioid endometrial cancer typically expresses the classical progesterone receptor (PGR). More recently it has been shown that some tumors also express non-classical progesterone receptors, such as progesterone receptor membrane component 1 (PGRMC1). PGRMC1 is a single transmembrane spanning protein that purportedly functions as a non-classical progestin receptor [8-11]. Despite its name, PGRMC1 was first isolated in microsomal fractions derived largely from the endoplasmic reticulum of liver cells [12,13]. PGRMC1 is also expressed in the plasma membrane [14,15] and nucleus [14-16].

Given the abundant and endocrine regulated expression of PGRMC1 in the endometrium [10,14,17-20], the observation that PGRMC1 expression is elevated in various cancers such

as ovarian and breast [21-26], and that P4 elicits actions in cells that lack expression of the classical PGR [9], the present study was undertaken to assess the role of PGRMC1 in endometrial cancer. Specifically, the objectives of this study were to: 1) evaluate PGRMC1 expression in endometrial cancer cell lines; 2) assess mitosis and cell death following treatment with P4 and/or chemotherapy in PGRMC1-intact and PGRMC1-deplete endometrial cancer cells; and 3) evaluate the onset of tumor formation and response to chemotherapy treatment *in vivo* following subcutaneous and intraperitoneal inoculation of PGRMC1-intact and PGRMC1-deplete endometrial cancer cells in immunocompromised mice.

Materials and methods

Development of PGRMC1-intact and PGRMC1-deplete Ishikawa cell lines

Ishikawa cells derived from the 3H12 clone which lack the classical PGR (i.e., EV3 Ishikawa cells) [27]; were cultured in phenol red free RPMI-1640 medium (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT), 100 U/ml penicillin G, 292 mg/ml L-glutamine, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B (Invitrogen, Carlsbad, CA) at 37 °C in a humidified atmosphere of 5% CO₂. These culture conditions were also used for Ishikawa cells expressing the classical progesterone receptor. The pLKO.1 vector harboring five different hairpin sequences for targeted knockdown of human PGRMC1 was packaged into lentiviruses at the Molecular Profiling Facility at the Massachusetts General Hospital Center for Cancer Research in association with the RNAi Consortium of the Broad Institute (Cambridge, MA) [28] as described in detail [29]. Control virus containing the pLKO.1 vector harboring a hairpin sequence (TRCN0000061298) for PGRMC2 was also generated. The PGRMC2 hairpin was ineffective at knocking down PGRMC1 or PGRMC2 and thus served as an effective control (i.e., PGRMC1-intact) for PGRMC1-deplete cells (see Fig. 2B and Supplementary Fig. S1). Infection titers were first established by infecting HEK293T cells grown on 96-well microtiter plates with 25 µl of diluted transfected supernatants containing lentiviral particles and 25 µl polybrene (Sigma; 48 mg/kg). The estimated multiplicity of infection for each virus was 1-2, which resulted in most transformed cells containing no more than one viral integrant [29]. The Ishikawa cells were then transformed using conditions as determined in HEK293T cells. After 24 h, culture medium containing viral particles was removed and cells demonstrating stable integration of the respective plasmids were selected by culturing cells for 72 h in puromycin (2 µg/ml). PGRMC1 levels were determined by RT-PCR and Western blot analysis upon expansion of selected clones. Subsequent cell lines used for experiments are hereafter referred to as PGRMC1-intact and PGRMC1-deplete Ishikawa cells.

Cell culture experiments

For assessment of apoptosis in response to chemotherapeutic stress, Ishikawa cells were rinsed with and converted to serum free medium one day prior to each experiment. PGRMC1-intact and PGRMC1-deplete cell lines were seeded in triplicate at equal densities (1×10^5 cells/well) in 24 well culture plates. At 50% confluence, PGRMC1-intact and PGRMC1-deplete cells lacking the classical progesterone receptor were treated with vehicle (0.03% ethanol), doxorubicin (Dox; 2 µg/ml, Alexis Biochemicals, San Diego, CA), P4

(1 μ M), or P4 for 30 min followed by Dox. The number of cells showing evidence of nuclear condensation or fragmentation was recorded as a percent of the total cells counted following fixation with 4% paraformaldehyde and Hoechst staining as previously described [30]. For evaluating the effects of P4 treatment on mitosis, PGRMC1-intact and PGRMC1-deplete cells were again cultured to 50% confluence, converted to serum free conditions as before and treated with P4 (0, 1, 10, 100, or 1000 nM) for 6, 24, 48 or 72 h. Following fixation and Hoechst staining the number of mitotic cells was recorded as a percentage of the total cells counted in five fields of view.

RNA isolation and RT-PCR

Total RNA was isolated using TriReagent from two lines of Ishikawa cells that vary in expression of the classical PGR (Sigma Chemical Co., St. Louis, MO). Samples were subjected to DNase I digestion (RQ1 RNase-free DNase; Promega, Madison, WI) to eliminate potential genomic DNA contamination. cDNA was synthesized using SuperScript II reverse transcriptase and oligo-dT primer (Life Technologies, Carlsbad, CA). Expression of various known and purported progesterone receptors was assessed by conventional RT-PCR using primer sets shown in Table 1. Each PCR product was sequenced to confirm specific amplification of the target gene. A negative control (i.e. mock reverse transcriptase) was also included for each mRNA sample in which reverse transcriptase was omitted to further confirm the absence of genomic DNA contamination. RT-PCR was also used to assess PGRMC1 mRNA expression in Ishikawa cells infected with lentivirus to knock down *PGRMC1* expression.

Western blot analysis

Protein lysates were collected from Ishikawa cells and six other endometrial cancer cell lines for Western blot analysis as described in detail [31]. After electrophoretic separation using the NuPage system (Invitrogen, Carlsbad, CA), proteins were transferred (30 V, 1 h) onto polyvinylidene difluoride membranes. Nonspecific binding was blocked with 5% fat-free milk in PBST buffer (0.1% Tween 20 in PBS) for 1 h at room temperature. PGRMC1 antibody was diluted in PBST with 5% fat-free milk and applied to membranes for overnight incubation at 4 °C. Membranes were then washed (3 $\times$ 10 min each) in PBST buffer and incubated with biotin conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA; 1:2500 dilution) for 1 h at room temperature. Membranes were washed in PBST as before, and bound antibody was detected using enhanced chemiluminescent reagents based on the manufacturer's recommendations (Amersham, Piscataway, NJ). Control blots were also completed in which primary antibody was omitted. To verify equal protein loading, membranes were then stripped [1M glycine (pH 2.5), 1 h, 37 °C] and reprobed with betaactin antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:1000 dilution).

Immunocytochemistry

PGRMC1-intact and PGRMC1-deplete Ishikawa cells were cultured in microscope slide culture chambers. Cells were fixed with 4% paraformaldehyde for 10 min at 4 °C and permeabilized with 100% methanol for 10 min at -20 °C. Cells were incubated in blocking

solution containing normal donkey serum and bovine serum albumin for 1 h at room temperature. PGRMC1 antibody (Sigma Aldrich, St. Louis, MO) was applied at 1:1000 and allowed to incubate overnight at 4 °C. Alexa 546 donkey anti-rabbit (Life Technologies, Carlsbad, CA) secondary antibody was applied at a dilution of 1:2000 for 1 h. Cells were washed 3 times with PBS, DAPI was applied in mounting medium, and cells were imaged.

Development of human endometrial xenograft tumors in NOD-SC/D and nude mice

All animal studies were approved by Institutional Animal Care and Use Committees at Washington State University or Massachusetts General Hospital. For generating subcutaneous tumors, 2×10^6 PGRMC1-intact or PGRMC1-deplete Ishikawa cells were suspended 1:1 in PBS/Matrigel® (BD Biosciences) and subcutaneously injected into the right and left dorsal flank of 6-week old female NOD/SCID mice (n=8 each group). Tumor growth was measured every third day externally with calipers. Tumor growth was calculated using a modified ellipsoidal equation for determining tumor volume (V): $V = [\text{length} \times (\text{width}^2)]/2$ [32]. Tumors were allowed to reach 10 mm³. Following pilot studies designed to optimize chemotherapy dosing and timing schedules, paclitaxel (15 mg/kg) (Sigma Chemical Company, St. Louis, MO) was administered intraperitoneally (IP) every fifth day for 3 cycles in a final volume of 200 µl, followed after an interval of 30 min by CARBOplatin (50 mg/kg) (Mayne Pharma Pty Ltd., Victoria, Australia) IP, in a final volume of 200 µl to half of the PGRMC1-intact and PGRMC1-deplete xenografted mice. The remaining mice received equivalent concentrations of vehicle (ctemophor EL® (BioChemika, St. Louis, MO):ethanol, 1:1) and 0.9% saline (Hospira Inc., Lake Forest, IL). Mice were weighed before drug administration and every 2 days until sacrifice. Mice were exposed to CO₂ and cervically dislocated five days after the final treatment. At necropsy each tumor was exposed, excised and fixed in 4% paraformaldehyde and paraffin embedded.

To monitor the growth of tumors that developed after intraperitoneal inoculation of endometrial cancer cells into nude mice, PGRMC1-intact and PGRMC1-deplete cells were first transformed with GFP using a lentiviral system according to the manufacturer's recommendations (GenTarget; San Diego, CA). Next, 5×10^6 GFP labeled PGRMC1-intact or PGRMC1-deplete cells were injected intraperitoneally into nude mice (The Jackson Laboratories, Bar Harbor, ME; n = 3). Tumor growth was determined at six weeks post-inoculation by isolating and collectively weighing all GFP-positive tumors from each mouse.

Statistical analyses

All experiments were replicated a minimum of three times. Data are presented as the mean $\pm$ SEM. For *in vitro* and *in vivo* experiments, a Student's t-test was used for simple pairwise comparisons. One-way ANOVA followed by a Tukey's post-hoc test was used to assess the *in vitro* time course and dose response experiments. A two-way ANOVA was used to analyze *in vivo* data to identify time and treatment effects. Assignment of mice to each experiment and treatment groups was made randomly. Raw data were analyzed with GraphPad PRISM software (version 4.0; San Diego, CA). Regardless of statistical test used, mean values were considered statistically different when $p < 0.05$.

Results

PGRMC1 expression in endometrial cancer cell lines

PGRMC1 is more abundantly expressed in a variety of tumors than in corresponding non-cancerous tissues [21-26]. Fig. 1A shows expression of PGRMC1 protein in seven human endometrial cancer cell lines. Beta-actin was used as a reference point for normalization. PGRMC1 expression varies from one cell line to the next with the greatest expression found in RL95-2 cells and the least expression in Hec1B cells (Fig. 1B). Because we are interested in assessing non-classical progesterone receptor biology, we next compared expression of the three classes of progesterone receptors in two previously published Ishikawa cell lines that are either deficient in or express PRA and PRB [27,33]. As shown in Fig. 1C, conventional RT-PCR confirmed the lack of PRA and PRB expression in EV3 Ishikawa cells (Fig. 1C, lane 2) in contrast to cells stably transfected with PRA and PRB (Fig. 1C, lane 1 [27]). Both cell types expressed two members of the progesterone receptor membrane component (PGRMC) family in *PGRMC1* and *PGRMC2*, as well as three members of the PAQR family purported to be membrane progesterone receptors (ie., *PAQR5*, *PAQR7* and *PAQR8*). *PAQR8* expression is reduced in EV3 Ishikawa cells, which lack expression of the classical *PRA* and *PRB* receptors. Despite being shown previously to be regulated in the endometrium by steroid hormones [14] and fluctuating during phases of the estrous/menstrual cycles [14,17-20], basal *PGRMC1* and *PGRMC2* expression is not dependent upon the presence of PRA and PRB (Fig. 1C).

Generation of PGRMC1-intact and PGRMC1-deplete Ishikawa cells

In order to determine whether PGRMC1 mediates the actions of P4 in endometrial epithelial cells in the absence of classical progesterone receptors PRA and PRB, we developed EV3 Ishikawa cells with stable depletion of PGRMC1 for comparison with control cells that retain expression of PGRMC1. Here, PGRMC1-intact control cells and PGRMC1-deplete cells were generated using a lentiviral-based shRNA knockdown approach. As shown in lanes 3, 5 and 6 in the representative Western blot (Fig. 2A), PGRMC1 protein was dramatically knocked down by three of five shRNAs that target different regions of *PGRMC1* mRNA. For all subsequent experiments, clone 1-2 (TRCN0000222108; Fig. 2A, shRNA2) was selected given that PGRMC1 expression was reduced by an estimated 90% compared with vector treated cells (pLKO1). EV3 Ishikawa cells transformed with shRNA targeting *PGRMC2* mRNA were generated as PGRMC1-intact cells. Based on mRNA expression this *PGRMC2* shRNA was ineffective at knocking down either *PGRMC1* or *PGRMC2* (Supplementary Fig. S1). This cell line was used for all subsequent experiments and represents the PGRMC1-intact cell line. Immunocytochemistry was used to confirm retention of PGRMC1 in PGRMC1-intact cells (Fig. 2B) and knockdown of PGRMC1 in PGRMC1-deplete cells (Fig. 2C) where PGRMC1 localized predominantly to the cytoplasm (Fig. 2B). A negative control is shown in Fig. 2D.

PGRMC1 mediates the anti-mitotic and anti-apoptotic actions of progesterone

Because PGRMC1 was shown to mediate the anti-mitotic and/or cell survival actions of P4 in spontaneously immortalized granulosa cells [34], ovarian cancer cells [21,22,35] and human luteinized granulosa cells [36], we assessed the actions of P4 in PGRMC1-intact

EV3 Ishikawa cells. The percentage of mitotic cells was determined for both vehicle and P4 treated cells by counting the number of mitotic figures and establishing this as a percentage of total cells. Data are presented as a change in mitosis with respect to reference levels observed in vehicle treated cells. Progesterone (100 and 1000 nM) decreased mitosis by about 50% in PGRMC1-intact cells at 48 h ($p < 0.05$; Fig. 3A). This reduction in mitosis was first observed 24 h following provision of P4 and continued through 72 h (Fig. 3B). While PGRMC1-intact cells responded to P4 treatment (Fig. 4A), PGRMC1-deplete cells failed to show a similar reduction in mitosis at 48 h (Fig. 4A), indicating that PGRMC1 mediates the anti-mitotic actions of P4 in endometrial epithelial cells. Unlike reproductive cells of the ovary and uterus, mitosis of nonreproductive epithelial colorectal adenocarcinoma Caco2 cells was unaffected by P4 treatment (Fig. 4B) despite retaining expression of *PGRMC1* and *PGRMC2* (Fig. 4C).

As shown in Fig. 5A, treatment of PGRMC1-intact cells with the chemotherapeutic agent doxorubicin (Dox) for 48 h resulted in a significant increase in the percentage of cells undergoing apoptosis. This is in contrast to control (vehicle treated) cells in which less than 2% of the cells were apoptotic. Pretreatment of PGRMC1-intact cells for 30 min with P4 attenuated Dox-induced cell death by approximately 50% despite these cells completely lacking any expression of PRA and PRB. Importantly, depletion of PGRMC1 prevented P4 from attenuating Dox-induced apoptosis, indicating that PGRMC1 mediates the anti-apoptotic actions of P4 in EV3 Ishikawa cells (Fig. 5B).

PGRMC1 contributes to human endometrial tumor growth and chemoresistance in vivo

We next evaluated endometrial xenograft tumor growth characteristics of PGRMC1-intact and PGRMC1-deplete cells. All mice eventually generated tumors when inoculated with either PGRMC1-intact or PGRMC1-deplete cells. However, an upward growth trajectory was first observed in PGRMC1-intact tumors 23 days after flank injection compared with PGRMC1-deplete tumors that showed increased growth at 29 days post-inoculation (Fig. 6A), suggesting that PGRMC1 confers an increased capacity to develop tumors *in vivo*. To ensure that this observation was not due to the site of inoculation, tumor growth was also compared by injecting GFP labeled PGRMC1-intact and PGRMC1-deplete cells intraperitoneally. As shown in Fig. 6B, the mean PGRMC1-intact tumor mass was 5-fold larger ($p = 0.02$) than PGRMC1-deplete tumor mass following a six week incubation period. This was accounted for by a greater number of tumors collected from PGRMC1-intact inoculated mice. GFP-labeled tumors were easily identified in mice inoculated with PGRMC1-intact (Fig. 6C) and PGRMC1-deplete (Fig. 6D) cells.

Since PGRMC1 promotes chemoresistance in the presence of P4 *in vitro*, we next sought to determine the effects of PGRMC1 on chemoresistance *in vivo*. While subcutaneous PGRMC1-intact tumors responded poorly to combined paclitaxel/CARBOPlatin therapy as evidenced by an absence of decreased tumor volume (Fig. 7A), PGRMC1-deplete xenograft tumors were more responsive to treatment based on a steady decline in tumor volume during treatment compared with vehicle treated tumors (Fig. 7B). This observation is shown histologically in Fig. 7C-F, where PGRMC1-deplete tumors from chemotherapy treated mice (Fig. 7F) are visibly smaller than tumors from vehicle treated mice (Fig. 7C and E,

respectively) or PGRMC1-intact tumor treated with chemotherapy (Fig. 7D). Many phosphohistone-H3 positive (ie, mitotic) cells were observed in the growing, non-fibrotic coltical regions of both PGRMC1-intact (Fig. 7G) and PGRMC1-deplete (Fig. 7H) chemotherapy treated tumors taken at the end of the treatment protocol. Given that mitosis is observed in both tumor types and that chemotherapeutic treatments are most effective in cells undergoing mitosis, this suggests that both tumor types have the capacity to respond to chemotherapy treatment.

Discussion

PGRMC1 is expressed in all mammals, as well as orthologs in invertebrate species and yeast [8,37,38]. This indicates that PGRMC1 is evolutionarily conserved and may be involved in a number of vital cellular processes [8,39]. This concept is supported by findings that PGRMC1 is implicated in the regulation of cholesterol biosynthesis [38,40], chemical detoxification [41], gene transcription [16], stress responses [42,43], neuronal guidance [37], activation of mitogen-activated protein kinase pathway [44], immune cell function [45] and cell division [9, 44, 46]. Importantly, PGRMC1 mediates the actions of progesterone (P4) in granulosa cells where it serves to attenuate apoptosis and mitosis [9,15,16,46]. More recently, it has been suggested that PGRMC1 is the sigma-2 receptor, an opioid-like receptor that is significantly elevated in a number of distinct cancers, although this area of investigation remains controversial [47-49].

The present study reveals that P4 suppresses mitosis of endometrial epithelial cancer cells as has been shown for endometrial epithelial cells. This observation is consistent with P4 being used as a treatment for low-grade endometrial cancers. Moreover, the anti-mitotic action of P4 is mediated by PGRMC1 as demonstrated in PGRMC1 shRNA knockdown studies. The anti-mitotic actions of P4 appear to be restricted to cells from reproductive tissues that express PGRMC1 given that Caco2 colorectal cells remain mitotic in the presence of P4. This outcome cannot be explained by an absence of PGRMC1 in Caco2 cells since these cells express PGRMC1 at levels similar to PGRMC1-intact EV3 Ishikawa cells. Our interpretation is that cells of reproductive tissues harbor the appropriate metabolic machinery that interacts with PGRMC1 to elicit the observed anti-mitotic response and that Caco2, and likely other cells from non-reproductive tissues, lack these necessary interacting proteins. However, it is clear that PGR is not one of the proteins required to facilitate P4:PGRMC1 actions given that PGRMC1-intact EV3 Ishikawa cells lack PGR. Identification of PGRMC1 interacting proteins is now needed to help sort out the mechanism by which PGRMC1 mediates the anti-mitotic actions of P4.

The effects of P4 activation of PGRMC1 are not solely restricted to inhibiting mitosis, since this signaling pathway also inhibits apoptosis. Importantly, the anti-apoptotic action of P4 in Ishikawa cell lines was clearly demonstrated by using a lentiviral shRNA approach to generate stable PGRMC1 -deplete cells. Interestingly, cotreatment of PGRMC1-deplete cells with Dox and P4 resulted in an increase in cell death over Dox treatment alone in PGRMC1-intact cells, suggesting that PGRMC1 sits at the interface between cell survival and cell death and that P4 contributes to survival decisions. The exact mechanism by which PGRMC1 exerts its pro-survival actions remains to be determined, but it may function

through multiple pathways given that PGRMC1 localizes to several subcellular compartments including the endoplasmic reticulum, nucleus and plasma membrane.

The anti-apoptotic effect of P4:PGRMC1 signaling has been previously observed in ovarian cells. Progesterone was shown to inhibit rat ovarian granulosa and luteal cell apoptosis following serum deprivation, as well as apoptosis of spontaneously immortalized granulosa cells (SIGCs) [34]. Likewise, SKOV-3 ovarian cancer cells co-treated with P4 showed a significant reduction in cell death in response to the chemotherapeutic agent cisplatin versus cells treated with cisplatin alone [21,22,35]. A unifying theme in all of these studies is that each cell line lacks expression of the classical PGR, but expresses PGRMC1. This suggests that the actions of P4 are being mediated by an alternative signaling pathway distinct from PGR. While the Ishikawa cell line used in our studies does not express classical PGR, it does express several putative membrane progesterin receptors including PGRMC1. Furthermore, because PGRMC1 has been shown to serve as a *bona fide* P4 receptor in that it binds P4 at high affinity [34] and is required for the anti-apoptotic actions of P4, it may serve as a focal point for the development of adjuvant cancer therapies. That PGRMC1 is also elevated in breast [23], lung [47], colon [50] and thyroid [50] cancers suggests that such adjuvant therapy, if developed and useful, could have general application to cancers beyond gynecologic cancers.

The present studies confirm previous findings that inoculation of immunocompromised mice with EV3 Ishikawa endometrial cancer cells results in formation of tumors [27,33], and this was demonstrated in the development of both subcutaneous and intraperitoneal tumors. Interestingly, Hanekamp et al. determined that EV3 cells injected intraperitoneally not only develop tumors, but that these tumors could be stimulated to growth upon treatment with medroxyprogesterone acetate (MPA) [27]. This finding is significant given that some postmenopausal women receive hormone therapies that often contain progestogens to minimize the unopposed actions of supplemental estrogens given as part of the therapy. In the case of PGR negative endometrial cancers, particularly those that are advanced, poorly differentiated, and/or recurrent, it would then seem that exposure to progestogens may actually facilitate tumor growth and survival if such tumors express elevated levels of the anti-apoptotic factor PGRMC1 as is often observed in other cancers. This concept is supported by studies in breast cancer cells, which showed an increase in proliferation in response to various progestogens [25], as well as The Women's Health Initiative Study in which progestin used in combination with estrogen for attenuating postmenopausal symptoms was shown to increase the risk of developing breast cancer [51]. Clearly, additional studies are needed to tease apart the mechanism(s) by which PGRMC1 provides an enhanced survival capacity for tumorigenic endometrial cells that lack the classical PGR. Such mechanisms could involve sumoylation [52], modulation of gene expression [16, 52], regulation of cell cycle progression [44, 46] or association with other proteins such as PAQR7 [53], PAIRBP1 [54], or aurora kinase B [55].

To validate our *in vitro* data, xenograft tumors were developed to compare growth characteristics and response to chemotherapeutic stress in PGRMC1-intact and PGRMC1-deplete cells. Depletion of PGRMC1 attenuated tumor growth while increasing responsiveness to combined paclitaxel and carboplatin chemotherapy treatment. Most

chemotherapeutic strategies are more effective on cells that are highly proliferative. The failure of chemotherapy treatment in PG RMC1-intact tumor size is likely not due to the absence of proliferation. PGRMC1-intact and PGRMC1-deplete tumors retained growth potential given that mitotic cells were observed in both types of tumors. Our interpretation is that depletion of PGRMO makes cells more sensitive to stressors such as chemotherapy. This interpretation is supported by similar studies in ovarian tumors and ovarian, breast and now uterine cancer cells. These findings add to our prior efforts in which we demonstrated that PGRMC1 contributes to ovarian tumor growth and chemoresponsiveness [22]. As with endometrial tumors, ovarian tumors deficient in PGRMO grow more slowly and become sensitive to chemotherapy. Follow-up studies are now needed to determine if PGRMC1-intact and PGRMC1-deplete endometrial tumors respond differently to P4 as was shown here to be the case *in vitro*, as well as in ovarian xenograft tumors *in vivo* [23]. It will also be important to determine if PGRMC1 functions differently in tumors that retain versus those that lack PGR expression in response to P4 and/or chemotherapy treatment.

It is interesting that PGRMC1 expression is elevated in a number of different cancers including ovarian [23], breast [23-26], thyroid [50] and lung [47] cancer in comparison to corresponding normal tissues. As such, in addition to identifying PGRMC1 interacting proteins, an understanding of how PGRMC1 is regulated in these cancers is needed. PGRMC1 is induced by carcinogens such as dioxin [41], PGRMC1 expression in ovarian cancer cells may be regulated by microRNAs. The let-7 isoforms let-7i and miR-98 were shown to decrease expression of PGRMC1 in SKOV-3 ovarian cancer cells in response to P4 treatment [56]. A study by Liu et al. further supports this idea in which a MUC1 aptamer-let7i chimeric system was used to selectively deliver let7i miRNAs to OVCAR-3 cells [57]. The result was a decrease in PGRMC1 expression and concomitant increase in chemosensitivity to paclitaxel treatment. In conoboration with ovarian studies, a recent study involving the use of Ishikawa cells showed that miR-98 represses *Pgrmc1* expression [58]. Let-7i and miR-98 may therefore exert regulatory actions on PGRMC1 and this in turn may impact on a broader range of normal endometrial activities such as cell cycle control or life:death decisions, as well as the transition into and maintenance of hyperproliferative (e.g., cancer) or so-called P4 refractory diseases such as endometriosis.

In summary, this study demonstrates that P4 activated PGRMC1 suppresses mitosis and apoptosis in endometrial cancer cells that lack PGR and that the actions of P4 were lost following knockdown of PG PGRMC1. Upon generation of xenograft tumors, the presence of PG RMO conferred a preferential growth advantage over cells that lacked PGRMC1. PGRMC1-deplete xenograft tumors grew much slower than PGRMC1-intact tumors. It was further demonstrated that PGRMC1-deplete tumors were more sensitive to chemotherapy than PGRMC1-intact tumors. This outcome is likely explained by the anti-apoptotic actions of PGRMC1, whereby loss of PGRMC1 renders PGRMC1-deplete tumors more susceptible to the killing actions of chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

(A) Expression of PGRMC1 in seven different endometrial cancer cell lines as determined by Western blotting. β -actin was used as a loading control. (B) Relative expression of PGRMC1 in endometrial cancer cell lines following normalization versus β -actin. (C) RT-PCR expression analysis of PRA, PRB, PGRMC1, PGRMC2, PAQR7, PAQR8 and PAQR5 in EV3 Ishikawa cells stably transfected with PRA and PRB (transfected) or EV3 Ishikawa cells that lack expression of PRA and PRB (untransfected).

Fig. 2.

Generation of PGRMC1-deplete EV3 Ishikawa cells. (A) Western blot showing PGRMC1 expression in parental EV3 Ishikawa cells transformed with pLKO1 empty vector (lane 1) or five different lentiviruses harboring shRNAs that target different regions of the PGR/V/C1 mRNA (shRNAs 1-5). Because of greater than 9 knockdown efficiency, cells transformed with shRNA TRGI0000222 IOB (shRNA2) were used in subsequent experiments and referred to as PGRMC1-deplete cells. PGRMC1 expression is shown by immunocytochemistry in PGRMC1-intact cells (B) and PGRMC1-deplete cells (C). Immunocytochemistry performed using PGRMC1-intact cells in the absence of primary antibody served as a negative control (D, n = 3). Images taken at 400x.

Fig. 3. Effect of P4 treatment on PGRMC1-intact EV3 Ishikawa cell mitosis. (A) Dose response showing change in mitosis upon 48 h treatment with indicated P4 concentrations. (B) Comparison of change in mitosis between vehicle and P4 treated PGRMC1-intact cells at 6, 24, 48 and 72 h. * indicates a significant difference compared with vehicle treatment group within time ($p < 0.05$). All experiments were repeated $n = 4$ times.

Fig. 4.

(A) Comparison of change in mitosis in response to P4 in PGRMC1-intact and PGRMC1-deplete cells. (B) Shown is the lack of change in mitosis of colorectal epithelial cells treated with P4. (C) RT-PCR showing expression of PGRMC1 and PGRMC2 in Caco2 and PGRMC1-intact EV3 Ishikawa cells. Water was used as a negative control to confirm expression of PGRMC1 in Caco2 and EV3 cells. * indicates a significant difference compared with vehicle treatment group ($p < 0.05$). All quantitative experiments were repeated $n = 4$ times.

Fig. 5.

Effect of P4 treatment on EV3 Ishikawa cell apoptosis. (A) The percentage of apoptotic cells was determined by DAPI staining of cultured PGRMC1-intact EV3 Ishikawa cells in response to treatment with vehicle, P4 (1000 nM), doxorubicin (Dax. 2 μ g/ml) or P4 and Dax. (B) The percentage of apoptotic PGRMC1-intact cells (white bars) in response to Dax or Dax and P4 treatment in comparison to Dax and P4 treated PGRMC1-deplete cells. * indicates significant difference compared with Dax treated PGRMC1 intact cells ($p < 0.05$). All experiments were repeated at least $n = 3$ times.

Fig. 6.

Comparison of xenograft tumor growth in PGRMC1-intact and PGRMC1-deplete cells 20-32 days after subcutaneous inoculation (flank, A. n = 8) or 6 weeks after intraperitoneal inoculation (B. n = 3). Representative images (15x) showing intraperitoneal xenograft tumor bulk from GFP transformed PGRMC1-intact (C) and PGRMC1deplete (D) EV3 Ishikawa cells.

Fig. 7.

Response of subcutaneous PGRMC1-intact (A) and PGRMC1-deplete (B) EV3 Ishikawa cell xenograft tumors to treatment with vehicle (cremophor EL: ethanol mixed at 1:1 ratio) or combined paclitaxel (15 mg/kg) and CARBOplatin (50 mg/kg) treatment given three times at 5 day intervals (arrows). * represents significant difference compared with vehicle treatment within time ($p < 0.05$). Medial histological sections of PGRMC1-intact (C, D) and PGRMC1-deplete (E, F) subcutaneous tumors treated *in vivo* with vehicle (C, E) or chemotherapy (D, F). Scale bar for C-F, 3 mm. Based on immunohistochemical staining for phosphohistone H3 (brown), mitosis was not different in vehicle treated PGRMC1-intact (G) and PGRMC1-deplete (H) tumors at the end of the experiment. All experiments were repeated $n=3-4$ times.

Table 1

PCR primers.

Gene name	Primer sequences
<i>PRA</i>	ACAGAATTCATGACTGAGCTGAAGGCAAAGGGT ACAAGATCTCAAACAGGCACCAAGAGCTGCTGA
<i>PRB</i>	ACAGAATTCATGAGCCGGTCCGGGTGCAAG ACAAGATCTCCACCCAGAGCCCAGGTTT
<i>PCRM1</i>	ACCTGCTGCTGCTTGGCCTCTG CCTGGATGCATCTCTTCCAGC
<i>PCKMC2</i>	AGAAGCGGGACTTCAGCTTG TCCCATTCTCGAACACTCTCC
<i>PAQK7</i>	CGGATGATCCAGCTCTTCTC CGTGTGCAGAGGCTCATAGA
<i>PAQR8</i>	TACCTCACCTGCAGCCTTCT GCAACAGCCAGCACAAGATA
<i>PAQR5</i>	ACTATGGTGCCGTCAACCTC TCCCAGGTGTACGGATAAGC