Progesterone Receptor Membrane Component-1 Regulates the Development and Cisplatin Sensitivity of Human Ovarian Tumors in Athymic Nude Mice

John J. Peluso, Anna Gawkowska, Xiufang Liu, Toshi Shioda, and James K. Pru

Departments of Cell Biology (J.J.P., A.G., X.L.) and Obstetrics and Gynecology (J.J.P.), University of Connecticut Health Center, Farmington, Connecticut 06030; Department of Tumor Biology (T.S.), Molecular Profiling Laboratory, Massachusetts General Hospital Center for Cancer Research, Harvard Medical School, Charlestown, Massachusetts 02129; and Vincent Obstetrics and Gynecology Service (J.K.P.), Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114

To determine whether progesterone receptor membrane component 1 (PGRMC1) regulates the development and cisplatin (CDDP)-sensitivity of human ovarian tumors, PGRMC1 was depleted from a human ovarian cancer cell line, dsRed-SKOV-3 cells, using a short hairpin RNA knockdown approach. Compared with parental dsRed-SKOV-3 cells, the PGRMC1-deplete cells grew slower in vitro and did not show progesterone’s (P4) antiapoptotic effect. In fact, P4 induced apoptosis in PGRMC1-deplete cells in a dose-dependent manner. When transplanted into the peritoneum of athymic nude mice, parental dsRed-SKOV-3 cells developed numerous tumors, which were classified as either typical or oxyphilic clear cell tumors. CDDP increased the percentage of apoptotic nuclei in typical clear cell tumors and P4 attenuated CDDP-induced apoptosis. In contrast, the percentage of apoptotic nuclei in oxyphilic clear cell tumors was low (≈1%) and was not significantly affected by CDDP and/or P4. Compared with tumors derived from parental dsRed-SKOV-3 cells, PGRMC1-deplete tumors: 1) developed in fewer mice, 2) formed less frequently, 3) appeared smaller, and 4) resulted in fewer oxyphilic clear cell tumors. These PGRMC1-deplete tumors were not responsive to CDDP’s apoptotic effects. The failure to respond to CDDP could be due to their poorly developed microvasculature system as judged by percentage of CD31-stained endothelial cells and/or their increased expression of ATP-binding cassette transporters, which are involved in drug resistance. Taken together, these findings indicate that PGRMC1 plays an essential role in the development and CDDP sensitivity of human ovarian tumors. (Endocrinology 150: 4846–4854, 2009)

Ovarian cancer kills more women than all the other gynecological cancers combined and is the fourth leading cause of cancer death among women in the United States (1). Unfortunately, most ovarian cancers are detected in advanced stages in which the cancer cells have metastasized from the ovary (1). Treatment of patients with ovarian cancer consists of surgery to remove the ovary, uterus, and tumor(s). This is usually followed by platinum-based (carboplatin and cisplatin) chemotherapy (1). Despite these intense surgical and chemotherapeutic treatments, the ovarian cancer more often than not recurs. At this point the patients are given salvage chemotherapy and possibly debulking surgery to remove the tumors that are usually distributed throughout the peritoneum (1).

Clearly the present chemotherapeutic agents are useful but not sufficient to effectively treat ovarian cancer. To improve the effectiveness of traditional chemotherapy, a genetic approach needs to be developed that can be used either alone or in conjunction with traditional chemotherapeutic agents. A genetic approach needs to be developed that can be used either alone or in conjunction with traditional chemotherapeutic agents.
apy. The target of this type of gene-based treatment should be a gene that is highly expressed in ovarian cancers compared with normal tissue and essential for ovarian cancer cell proliferation and/or survival.

Based on these criteria, progesterone receptor (PGR) membrane component 1 (PGRMC1) appears to be an excellent candidate for the development of such a gene-based treatment for ovarian cancer. PGRMC1 is detected at relatively high levels in all ovarian tumors examined, and its expression is increased in more advanced stages of ovarian cancer (2). Moreover, immunohistochemical analysis demonstrated that virtually 100% of tumors cells express PGRMC1 (2). Finally, an analysis of microarray data obtained from NCI (http://discover.nci.nih.gov/cellminer) revealed that PGRMC1 mRNA was very abundant in all ovarian cancer cell lines that were examined. These findings make it clear that PGRMC1 is highly expressed in ovarian tumors.

PGRMC1 also fulfills the second criterion in that it regulates cell survival in both normal and cancerous cells (3). This statement is based on in vitro studies in which genetic approaches were used to either deplete or enhance PGRMC1 levels. These studies conclusively demonstrate that PGRMC1 inhibits apoptosis (3–5). PGRMC1’s actions are induced by exposure to progesterone (P4) (3–5). Importantly, serum P4 levels are elevated in patients with ovarian cancer compared with age-matched controls (6). P4 levels are also higher in the peritoneal fluid of ovarian cancer patients (7). These findings suggest that ovarian cancer cells synthesize P4. Therefore, in the presence of endogenous P4, PGRMC1 is likely to be continuously active and function to promote the growth and development of ovarian tumors in vivo.

The present studies were designed to test this hypothesis by transplanting ovarian cancer cells into the peritoneum of athymic nude mice because ovarian cancers normally form secondary tumors within the peritoneal cavity (8). Although our previous work with ovarian cancer was conducted using the human ovarian cancer cell line Ovar-3 cells, these cells could not be used for the proposed in vivo studies because they rarely form tumors when transplanted in the peritoneum (9). Thus, to test our hypothesis, we first had to confirm the in vitro effects of PGRMC1 depletion on another ovarian cancer cell line that was capable of forming peritoneal tumors (9–12). For the present studies, SKOV-3 cells were selected. Once we confirmed that in vitro SKOV-3 cells respond to cisplatin (CDDP) and P4 in a manner indistinguishable from Ovar-3 cells, this cell line was used to generate PGRMC1-deplete human ovarian tumors in athymic nude mice and their formation and development, responsiveness to CDDP and/or P4, microvasculature, and expression of genes involved in drug resistance were compared with those tumors generated by parental SKOV-3 cells.

Materials and Methods

SKOV-3 cell culture

Culture conditions and in vitro treatments

SKOV-3 cells were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM-F12 (Sigma Chemical Co., St. Louis, MO) supplemented with 5% fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin G, and 100 μg/ml streptomycin (Invitrogen, Grand Island, NY), at 37°C in a humidified atmosphere of 5% CO2, incubator. The SKOV-3 cells were plated in serum-supplemented medium for 24 h and then CDDP (0–60 μM) and/or P4 (0–1 μM) added to the cultures as described for each experiment.

Generation of a dsRed-expressing SKOV-3 cell line

One million SKOV-3 cells were plated in a 100-mm culture dish and cultured for 24 h. The cells were then transfected with pDsRed2-ER, an expression vector that encodes a variant of red fluorescent protein, using the Lipofectamine-based protocol provided by Invitrogen. SKOV-3 cells were selected by the addition of geneticin (800 μg/ml; Invitrogen). After 4 wk, most of the cells expressed dsRed as judged by their fluorescence when observed under a tetramethylrhodamine isothiocyanate filter set. The high dsRed-expressing SKOV-3 cells were isolated by fluorescence-activated cell sorting. These high dsRed-expressing SKOV-3 cells were then maintained in DMEM-F12 culture medium supplemented with 100 μg/ml geneticin. Before being used in an experiment, geneticin was removed from the medium.

Apoptosis

SKOV-3 cells were placed in serum-containing medium supplemented with CDDP or P4 as indicated. After 24 h apoptotic nuclei were detected by in situ DNA staining by adding 4',6'-diamino-2-phenylindole (DAPI) directly to the culture medium to yield a final concentration of 2 μg/ml. The cultures were incubated for 10 min at 37°C in the dark. After staining, the cells were observed under epifluorescent optics with the DAPI filter set. Cells with condensed or fragmented nuclei were stained intensely with DAPI and considered to be apoptotic (2, 5). At least 100 cells/culture well were counted and the percentage of apoptotic nuclei in each well determined.

Mitosis

Both parental and PGRMC1-deplete dsRed SKOV-3 (4 × 104 cells/ml) were plated in T-25 flasks to which a horizontal and three vertical axes were scribed on the bottom outer surface. After 24 h, the number of cells within the four areas that were formed at the intersection of the horizontal and each vertical axis was counted (13). After 48, 72, and 96 h of culture, the number of cells present in these same areas was determined. Cell proliferation was expressed as a fold increase in cell number over 24-h values.

Generation of a PGRMC1-deplete SKOV-3 cell line

Lentiviruses packaged with either the empty plKO.1 vector or plKO.1 plasmid harboring a hairpin sequence (TRCN0000062905) for targeted knockdown of human PGRMC1 (NC_000023) were produced 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) (14) as described in detail by Smith and Shioda (15). Titers were 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, and this resulted in most transduced cells containing no more than one virus integrant (15). dsRed SKOV3 cells were then infected using conditions as described for HEK293T cells. After 24 h, DMEM-F12 culture medium containing viral particles was removed, and cells demonstrating stable integration of the respective plasmids were selected by culturing cells for 48–72 h in puromycin (2 μg/ml). Upon expansion of selected clones, PGRMC1 levels were determined by Western blot analysis.

Detection of progestin binding proteins

RT-PCR

Total mRNA was isolated from SKOV-3 cells and cDNA generated as previously described (2). The presence of nuclear PGR, members of the membrane progestin receptor (mPR) family, which are also known as progestin and adipQ: receptors (PAQRs), and PGRMC1 was then assessed by conducting 35 cycles of PCR (denaturation phase of 40 sec at 94°C; an annealing phase of 40 sec at 58°C and an extension phase of 60 sec at 72°C). The specific forward and reverse primers used to detect mRNA that encodes each progestin binding protein were: PGR, GTG-CAGGTGGAGACAGCT, TTTGCCCTICAGAAGCGGAC (213 bp); PGRMC1, GGCCTGATCGGAAGCCTG, TAATTCTTTTGCGGACT (202 bp); mPRα (PAQR7), GCGTTCTCTGAGCGCCCTACATCTATG, CAGACGCTTGGTGCCAGACATTAC (100 bp); mPRβ (PAQR8), GTCAACCTCTGCGACCCATT, GTGGGAGAGCTCTGACTTGG (bp 192); mPRγ (PAQR5), CAGCTGTCTTCCAGGGTCTGGTGTAGTCTCTG, GGACAGAAGTATGGGCA (182 bp). The primers used to detect PGR and PAQRs were based on the validated primers used by Misao et al. (16) and Hanna et al. (17), respectively. We validated the primers for PGRMC1 by sequencing the PCR product. These primers were identified using Primer 3 software (http://frodo.wi.mit.edu/) except for the primers for PGR, which were taken from Misao et al. (16).

Immunocytochemical detection of PGRMC1

Immunocytochemistry was used to assess the localization of PGRMC1 in paraformaldehyde-fixed SKOV-3 cells. The SKOV-3 cells were plated on coverslips, cultured at least 24 h, fixed with 4% paraformaldehyde at 4°C, and then incubated with 0.1% Triton X-100. PGRMC1 was localized using the NT-PGRMC1 antibody (4) and an Alexa Fluor 488-goat antirabbit IgG (1:100) as the secondary antibody as previously described (2, 18). The coverslips were observed under epifluorescent optics with the fluorescein isothiocyanate filter set to detect PGRMC1.

For Western blotting, SKOV-3 cells were lysed in buffer [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% Nonidet P-40] supplemented with protease, protein kinase, and protein phosphatase inhibitor cocktails (Sigma) and the lysates analyzed by Western blot as previously described (19) using either the NT-PGRMC1 antibody (1:5000) or a pan-actin antibody (1:1000; NeoMarkers, Fremont, CA). As negative controls, nonimmune IgG was used in place of the primary antibody.

Generation and characterization of ovarian tumor xenographs

Cellular transplantation

For these studies, 5-wk-old athymic nude female mice were purchased from Charles Rivers Laboratories (Wilmington, MA). The mice were injected ip with either 10 million parental dsRed-SKOV-3 cells (n = 36 mice) or 10 million PGRMC1-deplete dsRed-SKOV-3 cells (n = 26) as described by Chaudhuri et al. (20). After 5 wk in groups of six to eight, the mice were injected with a vehicle (control), CDDP (8 mg/kg body weight ip), P4 (1 mg/0.1 ml of corn oil sc), or both CDDP and P4. The 8 mg/kg CDDP dose has been previously shown to be effective in inducing cellular death within ovarian cancer xenographs (21, 22). A 1 mg/0.1 ml P4 sc injection was previously shown to be effective in modulating uterine function (19) and was the basis for selecting this dose for treating mice with ovarian cancer xenographs.

Three days after the CDDP/P4 injections, the mice were exposed to CO2 and then cervically dislocated. At autopsy the abdominal cavity was exposed and the tumors counted by observing them under a dissecting microscope under epifluorescent optics. A tumor was defined as an isolated mass that possessed dsRed fluorescence and was approximately 0.2 mm in diameter or greater. The tumors were then removed from the surrounding tissue and fixed in 10% formalin. All tumors from each mouse were placed in the same vial of fixative and embedded in the same paraffin block. This protocol was approved by the Animal Care Committee of the University of Connecticut Health Center.

Histological and immunohistochemical analyses

Five-micrometer sections were taken from each paraffin tumor block. Tumors were identified in hematoxylin and eosin-stained sections by their unique morphological features. That these tumors were derived from the dsRed-SKOV-3 cells was confirmed by immunohistochemical detection of dsRed. This was done using the anti-red fluorescent protein/DsRed antibody (catalog no. PM005) and protocol provided by MBL International Corp. (Woburn, MA). The classification of tumors as either typical or oxyphilic clear cell tumors was confirmed by a medical pathologist.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay

Apoptotic cells were detected by TUNEL assay in which formalin-fixed paraffin-embedded tumors were sectioned at 5 μm and stained using the Apoptag peroxidase in situ kit staining according to the manufacturer’s instructions (Chemicon, Temecula, CA). The sections were then counterstained with methyl green. The apoptotic nuclei were revealed by a reddish brown stain. Sections of 424 tumors taken form a total of 62 mice were examined.

The percentage of apoptotic nuclei within a tumor was determined by first counting the number of apoptotic (TUNEL positive) cells within a cross-section of each tumor. The cross-section of each tumor was then scribed and the area of the tumor determined using iVision software (BioVision Technologies, Exton, PA). Similarly the mean area of 100 tumor cells was determined. The number of cells within a given section of the tumor was estimated by dividing the area of the tumor by the average.
primary antibody was absent and replaced with IgG (negative control) or present, 

Endocrinology, November 2009, 150(11):4846 – 4854 endo.endojournals.org

PGRMC1 in SKOV-3 cells (E). The 

shown. The Western blot and immunocytochemical analyzes confirm the presence of 

progestin/adipoQ receptor family (mPR

1000 nM P4 on apoptosis of both parental and dsRed-SKOV-3 cells is shown in C. In this and 

subsequent graphs, values are shown as means ± 1 SE. *, Values that are different from 

controls (P < 0.05). D, PCR amplicons of mRNAs that encode the PGR and members of the 

progestin/adipoQ receptor family (mPRA, -β, and -γ) and PGRMC1 are shown. As a positive 

control for PGR expression, mRNAs isolated from a human granulosa/luteal cell line, GL5 is 

shown. The Western blot and immunocytochemical analyzes confirm the presence of 

PGRMC1 in SKOV-3 cells (E). The — and + indicate lanes of a Western blot in which the 

primary antibody was absent and replaced with IgG (negative control) or present, 

respectively.

area of a tumor cell. The percentage of apoptotic nuclei was then calculated.

CD31 staining

Five-micrometer sections were taken from tumors from each animal. Before staining the sections were deparaffinized, incubated with sodium citrate buffer at 95 C for 20 min and incubated in 0.3% hydrogen peroxide in methanol for 30 min at room temperature to quench endogenous peroxidase activity. The sections were stained for the presence of CD31 using the anti-CD31 antibody (catalog no. SC 1506, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) according to the procedure provided by the manufacturer.

To estimate the percentage of CD31-positive cells, the CD31-stained area within an individual tumor was determined using the threshold function of the iVision software. The area of the entire tumor was determined using the iVision software and the percentage of CD31 for each tumor calculated.

Gene expression

In this study, two to three mice with tumors derived from either parental or PGRMC1-depleted dsRed SKOV-3 cells were removed, dissected free from the adhering adipose tissue, and placed in the RNA isolation solution, RNA later (QIAGEN, Inc., Valencia, CA). These mice were not treated with either CDDP or P4 and this experiment was repeated twice. The mRNA from each replicate was isolated and the amount of mRNA that encoded 84 genes associated with cancer drug resistance and metabolism determined by real-time PCR using RT² Profiler PCR array provided by SABiosciences (Frederick, MD). Each sample was run in duplicate.

Statistical analysis

All experiments were repeated two to three times. Each apoptosis experiment was conducted in triplicate. When appropriate, the data were pooled to generate means ± se and analyzed by a one-way ANOVA followed by a Student-Newman-Keuls test. The differences in percentage of mice that developed tumors and the percentage of oxyphilic clear cell tumors were assessed by χ² analysis. The differences in gene expression (i.e. mRNA levels) were assessed using the software provided by SABiosciences. Regardless of the statistical test used, values were considered to be significantly different if P ≤ 0.05.

Results

In the presence of serum, CDDP induced SKOV-3 cells to undergo apoptosis in vitro in a dose-dependent manner (Fig. 1A). The apoptotic effect of CDDP was attenuated by P4 treatment in a dose-dependent manner (Fig. 1B). P4’s anti-apoptotic action was observed in both parental SKOV-3 cells and SKOV-3 cells that expressed the fluorescent protein, dsRed (Fig. 1C).

Although the mRNA that encodes PGR was not detected in SKOV-3 cells, PCR analysis revealed the presence of mRNAs that encode three members of the progestin/adipoQ receptor family (mPRA, -β, and -γ) as well as PGRMC1 (Fig. 1D). The expression of PGRMC1 was confirmed by Western blot analysis and immunocytochemistry (Fig. 1E). The Western blot detected two specific bands, which represent the monomeric and presumably dimeric forms of PGRMC1. Moreover, PGRMC1 was localized to the nucleus, cytoplasm, and segments of the plasma membrane (Fig. 1D).

Having shown that the dsRed SKOV-3 cells responded to both CDDP and P4 in a manner indistinguishable from the parental SKOV-3 cells, the dsRed SKOV-3 cells were injected into the peritoneal cavity of athymic nude female mice. After 5 wk, ovarian tumors derived from the dsRed SKOV-3 cells were detected by their red fluorescence (Fig. 2, A and B). Histological examination of these tumors revealed two types of tumors (Fig. 2C). The first type was classified as typical clear cell tumors (Fig. 2D), which are known to develop after SKOV-3 cell transplantation. The second type was classified as oxyphilic clear cell tumors (Fig. 2E). That both tumor types were derived from dsRed SKOV-3 cells was confirmed by immunohistochemical localization of dsRed (Fig. 3A).

Three days after a single ip injection of CDDP, an increase in the percentage of cells undergoing apoptosis was
observed in the typical clear cell tumors (Fig. 3, B and C). In contrast, apoptotic nuclei were rarely observed in oxyphilic clear cell tumors, and CDDP induced a slight but not statistically significant increase in the percentage of apoptotic nuclei (Fig. 3, B and C).

To determine whether PGRMC1 is required for P4 to attenuate CDDP’s killing effects in vivo, a PGRMC1-deplete dsRed SKOV-3 cell line was generated. That PGRMC1 was successfully depleted was demonstrated by Western blot analysis (Fig. 4A). Moreover, in vitro studies revealed that PGRMC1-deplete SKOV-3 cells had a slower rate of cell proliferation (Fig. 4B). Unlike its effect on parental dsRed-SKOV-3 cells that stably express the empty vector (Fig. 4C), P4 did not block CDDP-induced apoptosis and actually promoted apoptosis in PGRMC1-deplete dsRed-SKOV-3 cells (Fig. 4C). This apoptotic effect of P4 was dose dependent with an effective dose of 10 nM (Fig. 4D).

After injecting PGRMC1-deplete dsRed-SKOV-3 cells into the peritoneum of athymic nude female mice, only 80% of the mice formed tumors compared with 100% of the mice injected with parental dsRed SKOV-3 cells (Fig. 5A). In the mice with PGRMC1-deplete tumors, the number of tumors was reduced (Fig. 5B), and the tumors appeared smaller than tumors derived from parental SKOV-3 cells. Finally, the percentage of oxyphilic clear cell tumors derived from PGRMC1-deplete SKOV-3 cells were reduced compared with parental SKOV-3 cells (Fig. 5C).

Interestingly, typical clear cell tumors derived from parental SKOV-3 cells responded to CDDP and P4 in a manner that was similar to the in vitro responses of parental SKOV-3 cells (compare Fig. 1B with Fig. 6A). However, typical clear cell tumors generated from PGRMC1-deplete SKOV-3 cells had a very low rate of apoptosis, with the
mean of the vehicle control less than 2%. Although typical clear cell tumors derived from PGRMC1-deplete SKOV-3 cells appeared to respond to CDDP and P4 in a manner similar to that of typical clear cell tumors derived from parental SKOV-3 cells, their responses to CDDP and P4 were not statistically different from vehicle control ($P < 0.05$) (Fig. 6A).

To assess microvasculature, sections of these tumors were stained for CD31, a marker protein for the endothelial cells of the microvasculature (23). This staining revealed that CD31-stained endothelial cells comprised $2.8 \pm 0.8\%$ of the area of tumors derived from parental SKOV-3 cells (Fig. 6B) but only $0.4 \pm 0.1\%$ ($P < 0.05$) of the area of tumors derived from PGRMC1-deplete SKOV-3 cells (Fig. 6C). The same CD31 staining pattern was observed in both typical and oxyphilic clear cell tumors. Consistent with the reduced microvasculature, the gene expression analysis revealed a slight but statistically significant increase in the mRNA levels of aryl hydrocarbon receptor nuclear translocator (ARNT) and its binding partner, hypoxia-inducible factor 1alpha (HIF1A) as well as the mRNAs that encode several ATP-binding cassette (ABC) transporters genes (Fig. 6D).

### Discussion

The present in vitro studies confirm our previous work with OvCar-3 cells (2) that P4 attenuates CDDP-induced apoptosis and that this action is mediated through PGRMC1. Similar in vitro findings have been observed for breast (24–27) and endometrial cancer cell lines (27), thereby supporting a generalized role for P4 activation of PGRMC1 in cancer cell resistance to chemotherapeutic agents.

To truly test this concept, PGRMC1’s role in tumor development must be assessed in vivo. The present studies are the first to demonstrate in any in vivo model that PGRMC1 plays an important role in tumor formation. This is clearly demonstrated by the observations that PGRMC1-deplete ovarian cancer cells form tumors in fewer mice, and in those mice that form tumors, the number of tumors are reduced by 55%. Similarly, our preliminary studies with xenograft tumors derived from PGRMC1-deplete endometrial cells (IKLV Ishikawa cells) and breast cancer cells (MDA-MB-231) reveal that they grow more slowly than tumors derived from their respective parental cell lines (27). That PGRMC1 functions similarly in the formation of tumors from dif-
different tissue sources provides compelling *in vivo* evidence of a fundamental importance of PGRMC1.

The present studies confirm the findings from the laboratories of Vanderhyden and colleagues (9) and Murdoch and colleagues (10–12) that transplanting SKOV-3 cells into athymic nude female mice results in the development of clear cell tumors. The present studies also reveal the presence of oxyphilic clear cell tumors. These tumors are a specialized type of clear cell tumor that is characterized by cells with highly eosinophilic cytoplasm containing glycogen (28). Other than their morphological features, very little is known about these tumors. The present studies provide some functional insight into these tumors by demonstrating that they are less responsive to CDDP and P4 than typical clear cell tumors. Why CDDP and P4 are unable to significantly influence the rate of apoptosis in this subset of ovarian clear cell tumors remains to be elucidated.

Whereas the *in vivo* studies clearly demonstrate that PGRMC1 influences tumor development, its mechanism of action remains unknown. The *in vitro* studies suggest two possible mechanisms. First, PGRMC1 could promote or facilitate cell proliferation because PGRMC1-deplete SKOV-3 cells have a reduced rate of proliferation *in vitro*. A second mechanism could be that in the absence of PGRMC1 endogenous P4 induces apoptosis. This mechanism is consistent with the *in vitro* responses. Moreover, the apoptotic action of P4 is observed at 10 nM, which approximates serum levels of P4 (10).

The question now becomes why in the absence of PGRMC1 does P4 induce apoptosis *in vitro*. The answer to this question is unknown but may relate to the presence of other progestin binding proteins. It is important to appreciate that SKOV-3 cells express not only PGRMC1 but also three members of the progestin/adipoQ receptor family. These progestin/adipoQ receptors have binding constants in the nanomolar range (17). Thus, in the absence of the PGRMC1, it is possible that P4 acts through these progestin/adipoQ receptors to trigger an apoptotic cascade. This concept is based on the observations that P4 activation of progestin/adipoQ receptors results in an increase in MAPK activity (17) and increases in MAPK activity can promote apoptosis in breast cancer cells (29) as well as ovarian cells (23). Conversely, in the presence of PGRMC1, P4 suppresses MAPK activity (30), and the suppression of MAPK is an essential component of P4’s antiapoptotic action (30). Therefore, a decrease in the ratio of PGRMC1 to progestin to adipoQ receptor could result in a prolonged and inappropriate increase in P4-regulated MAPK activity, which would lead to apoptosis. This hypothesis merits further consideration.

Although in the absence of PGRMC1 P4 induces apoptosis, why then does P4 fail to induce apoptosis in PGRMC1-depleted tumors? The most likely answer is that endogenous serum P4 acts on transplanted PGRMC1-deplete SKOV-3 cells and induces their apoptosis, thereby attenuating their ability to form tumors. Those PGRMC1-deplete SKOV-3 cells that survive and form tumors are likely to be structurally distinct with altered gene expression profiles, and these differences could alter their responsiveness to serum levels of P4 as well as CDDP.

One observed structural difference is in the microvasculature. The microvasculature of PGRMC1-deplete tumors is only 14% of that of tumors derived from parental SKOV3-cells. In retinal glial cells that express high levels...
of PGRMC1, P4 stimulates the expression of vascular epithelial growth factor (VEGF) (31). This suggests that P4 activation of PGRMC1 increases VEGF expression (31). If PGRMC1 enhances VEGF expression in ovarian tumors, then this would account in part for the reduced microvasculature of tumors derived from PGRMC1-deplete SKOV-3. More importantly, the reduced vascular network of PGRMC1-deplete tumors could adversely affect the delivery of CDDP and P4, which would result in a reduced responsiveness to these agents (32).

Changes in gene expression also characterize tumors derived from PGRMC1-deplete SKOV-3 cells. Interestingly, mRNA levels for ARNT and its binding partner HIF1A are increased in PGRMC1-deplete tumors. These two proteins form a complex that functions as the transcription factor, hypoxia-induced transcription factor-1, which in turn regulates the expression of numerous genes including the ABC transporters (32). The ABC transporters function to export various chemotherapeutic agents from the cells, thereby increasing their resistance to chemotherapy (33, 34). Importantly, PGRMC1 depletion results in tumors with a 3- to 4-fold increase in the level of ABCC2 mRNA. This ABC transporter is highly effective in expelling CDDP from cells (35). Thus, this large increase in the expression of ABCC2 together with the reduced microvasculature could account in part for the relative insensitivity to CDDP of PGRMC1-deplete tumors compared with tumors derived from parental SKOV-3 cells.

In summary, the present studies demonstrate that PGRMC1 regulates not only the growth and CDDP sensitivity of cultured SKOV-3 cells but also ovarian tumors derived from these cells. PGRMC1 may facilitate ovarian tumor formation in part by promoting mitosis, cell survival, and microvasculature development. In the absence of PGRMC1, the ovarian tumors are more resistant to CDDP. This increase in CDDP resistance could be due to reduced microvasculature and/or the overexpression of ABC transporters.

Acknowledgments

The authors thank Dr. Ling Zhang for her technical work involved in developing the PGRMC1-deplete SKOV-3 cell line and Ms. Nancy Ryan for the histological and immunohistochemical staining. The authors also acknowledge Drs. Wehling and Losel (University of Heidelberg-Mannheim, Mannheim, Germany) for providing the antibody to PGRMC1 and Dr. Melinda Sanders for consulting in the histological character of the ovarian tumors. We also thank Dr. Kevin Claffey for his helpful suggestions and the use of his stereomicroscope.

References

5. Peluso JJ, Romak J, Liu X 2008 Progesterone receptor membrane component-1 (PGRMC1) is the mediator of progesterone’s anti-apoptotic action in spontaneously immortalized granulosa cells as revealed by PGRMC1 small interfering ribonucleic acid treatment and functional analysis of PGRMC1 mutations. Endocrinology 149:534–543
15. Smith S, Shioda T 2009 Advantages of COS-1 monkey kidney ep-