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RESEARCH ARTICLE

PGRMC1 contributes to doxorubicin-induced chemoresistance in MES-SA uterine sarcoma

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Abstract Chemotherapy is one of the major categories of medical oncology and a primary tumor treatment; however, the effectiveness of chemotherapy is restricted by drug resistance. Overcoming resistance to chemotherapy and investigating molecular targeted therapies are challenges currently faced during resistance management. Progesterone receptor membrane component 1 (PGRMC1) is an adapter protein mediating cholesterol synthesis, steroid signaling, and cytochrome p450 activation. Attention has recently focused on the role of PGRMC1 in cell survival, anti-apoptosis, and damage response. In the present study, we used knockdown and overexpression approaches in the following set of uterine sarcoma models to further evaluate the role of PGRMC1 in drug resistance: the doxorubicin-sensitive MES-SA cells and the doxorubicin-resistant MES-SA/DxR-2 μM and MES-SA/DxR-8 μM cells (with different levels of doxorubicin resistance). PGRMC1 repressed doxorubicin-induced cytotoxicity and exhibited an anti-apoptotic effect; it also promoted cell proliferation and cell cycle progression to the S phase. Of note, PGRMC1 overexpression led to the epithelial–mesenchymal transition (EMT) of the sensitive MES-SA cells, thus facilitating their migration and invasion. The combination of PGRMC1 knockdown and the P-glycoprotein inhibitor verapamil significantly decreased the viability of P-glycoprotein-overexpressing MES-SA/DxR-8 μM cells after doxorubicin treatment. Taken together, our results show that PGRMC1 contributed to chemoresistance through cell proliferation, anti-apoptosis, and EMT induction, leading to the suggestion that PGRMC1 may serve as a therapeutic target in combination with an inhibitor in different drug resistance pathways and indicating the usefulness of predictive resistance biomarkers in uterine sarcoma.

Keywords Drug resistant · Andriamycin · Programed cell death · Uterine cancer · Metastasis

Abbreviations

DAPI	4′,6-Diamidino-2-phenylindole
DTT	Dithiothreitol
FBS	Fetal bovine serum
Insig	Insulin-induced gene
MTT	3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide
PAIR-BP1	Plasminogen activator inhibitor 1 mRNA-binding protein

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PGRMC1	Progesterone receptor membrane component 1
Scap	SREBP cleavage activating protein
siRNA	Small interfering RNA

Introduction

Uterine sarcomas are relatively uncommon tumors that comprise approximately 3 % of all uterine cancer cases. They are associated with a high mortality rate due to the aggressive and recurrence characteristics of the tumor. The current lack of optional treatments and inconsistency in the identification of risk factors result from the rarity of the tumor and a wide diversity of histopathological results. In addition, very few reports on uterine sarcomas have been published due to their low occurrence rate and a shortage of biopsies for study. Patients with uterine sarcoma have a poor prognosis after surgical treatment; consequently, chemotherapy is a very important alternative treatment [1, 2]. However, drug resistance remains a major cause of chemotherapy failure despite efforts to develop alternative chemotherapeutic strategies. Consequently, alternative treatments to improve the effectiveness of chemotherapy need to be developed .

Doxorubicin (Adriamycin) is an anthracycline drug widely used in the treatment of various cancers, including lung, breast, ovarian, gastric, and uterine cancers [3–5]. It intercalates into DNA and stabilizes the topoisomerase II protein, thus halting DNA replication and transcription [6]. In addition, the doxorubicin-induced free radical further damages the cell membrane, DNA, and proteins, thus leading to apoptosis [7]. Although doxorubicin is an effective antineoplastic agent, in addition to its cytotoxic effect, resistance is the major limitation in its usage in chemotherapy. Biological mechanisms underlying doxorubicin-resistance include an enhanced activity and expression of membrane-embedded drug efflux transporters, such as ATP-binding cassette transporters [MDR1, also known as P-glycoprotein 1 (P-gp)], increased levels of intracellular detoxification enzymes, such as glutathione S transferase, and decreased levels of topoisomerase II expression [8, 9]. Although several drug resistance mechanisms have been investigated, drug resistance in emerging resistant cases is a complex cellular response. Thus, investigating additional unclarified resistance mechanisms is absolutely essential.

Because chemotherapy resistance is an integrated response, linking alterations in numerous proteins, the use of proteomic strategies to reveal chemoresistance-related proteins would appear to be appropriate. In an earlier proteomic study, we found that various proteins were involved in different drug resistance mechanisms, including asparagine synthetase and progesterone receptor membrane component 1 (PGRMC1, also abbreviated as mPR) [10]. PGRMC1 is a heme-binding cytochrome b_5-related protein containing predicted binding sites for Src homology 2 (SH2) and SH3 domains [11, 12]. PGRMC1 is involved in cholesterol synthesis through interaction with cytochrome p450 proteins, insulin-induced gene 1 (Insig-1), sterol regulatory element binding protein (SREBP) cleavage activating protein (Scap), and plasminogen activator inhibitor 1 mRNA-binding protein (PAIR-BP1) in normal cells [13]. Particularly in cancer cells, PGRMC1 is a multifunctional mediator involved in tumorigenesis, cell proliferation, anti-apoptosis, and damage response. PGRMC1 levels are highly elevated in colon, breast, lung, and ovarian cancer cells compared with corresponding nonmalignant cells [14–16]. Furthermore, PGRMC1 promotes tumor growth and proliferation in non-small cell lung cancer [17] and ovarian cancer [18]. PGRMC1 loss enhances progesterone (P4)-induced apoptosis [18]. In addition, PGRMC1 suppresses the apoptotic effect in breast [19] and ovarian cancers [16] by activating Akt phosphorylation [20, 21]. Similarly, the homolog of PGRMC1, Dap1, is critical for the damage-dependent survival of yeast cells [22]. PGRMC1 is induced during the late stage of ovarian cancer progression and participates metastasis [16]. Its depletion in lung cancer considerably decreases cell migration [17]. Although PGRMC1 is involved in the different developmental aspects of various cancers, from tumorigenesis to metastasis, the role of PGRMC1 in drug resistance in uterine sarcomas and the underlying mechanisms of this resistance have not been evaluated.

In this study, we used PGRMC1 knockdown and overexpression in self-established doxorubicin-induced resistant and original doxorubicin-sensitive uterine sarcoma cells to confirm the role of PGRMC1 in drug resistance. We found that PGRMC1 not only protected resistant cells from doxorubicin-induced p38-mediated apoptosis but also promoted cell cycle progression. In addition, PGRMC1 significantly induced the migration and invasion of noninvasive MES-SA cells. Furthermore, the combination of PGRMC1 depletion and verapamil, a P-gp inhibitor, could improve the sensitivity of resistant uterine sarcoma cells to doxorubicin. Based on results, we conclude that PGRMC1 is involved in doxorubicin-induced drug resistance and may serve as a therapeutic target against resistance in uterine sarcoma.

Materials and methods

Chemicals and reagents

Doxorubicin and verapamil were purchased from ACROS Organics N.V. (Geel, Belgium). Lipofectamine®

RNAiMAX Reagent and OPTI-MEM were purchased from the Invitrogen Corp., Life Technologies, Thermo Fisher Scientific, Waltham, MA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from the USB Corp. (Cleveland, OH, USA), and CellTiter Blue was purchased from the Promega Corp. (Madison, WI). P-gp, Glutathione S transferase, Topoisomerase II, α-Tubulin, PGRMC1, Bcl2 (an anti-apoptotic protein), Bax (a pro-apoptotic protein), poly(ADP-ribose) polymerase (PARP), caspase 9, caspase 3, phospho-ERK, cyclin A2, FoxM1, SNAIL, TWIST, and E-cadherin primary antibodies were purchased from Genetex (Hsinchu, Taiwan); phospho-p38, p38, phospho-Akt, and Akt were purchased from Cell Signaling Technology (CST; Beverly, MA); vimentin was purchased from Dako-Agilent Technologies (Glostrup, Denmark); cyclin D2 and cyclin E were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-mouse and anti-rabbit secondary antibodies were purchased from GE Healthcare Sverige AB (Uppsala, Sweden). Fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The annexin V FITC Apoptosis Detection kit was purchased from BD Biosciences (Franklin Lake, NJ). All chemicals and biochemicals used in this study were of analytical grade.

Cell lines and cell cultures

The uterine sarcoma cell line MES-SA was purchased from the American Type Culture Collection (Manassas, VA) and cultured in McCoy's 5a modified medium containing 10 % fetal bovine serum (FBS), L-glutamine (2 mM), streptomycin (100 μg/mL), penicillin (100 IU/mL) (all from the Gibco–Invitrogen Corp., Paisley, UK). Its doxorubicin-resistant partners, lines MES-SA/DxR-2 μM and MES-SA/DxR-8 μM, cells were both homogenetic with the MES-SA line. The resistant cell line was selected from the MES-SA/Dx5 cell line (ATCC) at the beginning of its doxorubicin treatment at one-fifth half-maximal inhibitory concentration (IC_{50}) for 48 h. Briefly, MES-SA/Dx5 cells were grown on culture medium containing doxorubicin and subjected to successive selection cycles of culture on medium containing increasing doxorubicin concentrations until the selected cells were able to grow in medium containing the desired specific concentration of doxorubicin with at least a 80 % cell survival rate for 2 weeks. The established MES-SA/DxR-2 μM and MES-SA/DxR-8 μM cells were cultured in the same medium supplemented with 0.2 and 0.8 μM doxorubicin, respectively, to maintain their resistance ability to these levels of doxorubicin. All cells were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2. MES-SA/DxR-2 μM and MES-SA/DxR-8 μM cells were returned for culture in complete McCoy's 5a modified medium at least 1 week prior to the experiment. The endometrial cancer ECC-1 cell line was obtained from Dr. Yung-Jen Chuang (Institute of Bioinformatics and Structural Biology and Department of Medical Sciences, National Tsing Hua University). Cells were maintained in RPMI-1640 medium containing 10 % FBS, L-glutamine (2 mM), streptomycin (100 μg/mL), penicillin (100 IU/mL) (Gibco-Invitrogen).

MTT and CellTiter Blue Cell Viability Assay

Cells were seeded into 96-well plates at a density of 7,000 cells/well. After an overnight incubation, the cells were exposed to different doxorubicin or verapamil concentrations for 48 h, following which the medium was removed and the cells incubated in MTT solution (1 mg/mL), 100 μL/well, at 37 °C for 4 h. The supernatant was then removed and 100 μl dimethyl sulfoxide (DMSO) was added per well to dissolve the insoluble formazan. The 96-well plates were shaken for 10 min and absorbance at 545 nm was measured by an ELISA reader. The CellTiter Blue Cell Viability Assay (Promega) was performed according to the manufacturer's protocol and the fluorescence measured in 560ex/590em using a Gemini fluorescence microplate reader (Molecular Devices, Sunnyvale, CA).

Immunoblotting assay

The effect of PGRMC1 on MES-SA, MES-SA/DxR-2 μM, and MES-SA/DxR-8 μM cells was validated using the immunoblotting assay. The detailed experimental procedure has been described previously [10]. All of these immunoblotting images were scanned by LAS 4000 (GE Healthcare, Little Chafont, UK). The antibodies used for immunoblotting are listed in the "Chemicals and reagents" section.

siRNA design, overexpression of stable clone, and transfection

The targeting sequences 5′-AAU UUG CGG CCU UUG GUC ACA UCG A-3′ and 5′-AGU GAA CUG AGA CUC CCA GUC ACU C-3′ against PGRMC1 were designed and synthesized by Invitrogen. Sequences with a similar GC content were used as the negative control against PGRMC1 (Invitrogen). MES-SA, MES-SA/DxR-2 μM, and MES-SA/DxR-8 μM cells were transfected with 60 nM PGRMC1 small interfering RNA (siRNA) or the corresponding scramble control (pGCsi-control) using the Lipofectamine® RNAiMAX transfection reagent (Invitrogen) following the manufacturer's instructions. All cell

lines were in 50 % confluence at the time of transfection. The Myc-DDK-tagged ORF clone of *Homo sapiens* PGRMC1 and its non-coding pCMV6-ENTRY vector were purchased from ORIGENE-GenDiscovery Biotechnology, Inc. (New Taipei City, Taiwan). The PGRMC1 cDNA was verified by sequencing. MES-SA cells were also transfected with PGRMC1 cDNA containing the vector or the empty vector (pcMV6) using the Lipofectamine® RNAiMAX transfection reagent (Invitrogen) following the manufacturer's procedure. After 48 h, the AMRESCO G418 antibiotic solution (AMRESCO LLC, Solon, OH) was added at concentration of 800 μg/mL. The populations of transfectants were isolated by several (sub)cultures and G418 exposures for 2 months and then further examined for PGRMC1 expression by immunoblotting. MES-SA cells demonstrating stable PGRMC1 transfection were maintained in G418 at a concentration of 400 μg/mL, but replaced in complete McCoy's 5a modified medium for culture at least 1 week prior to the experiment.

Overexpression of stable clone, lentiviral vector preparation, and infection

pLenti-IRES-Neo was purchased from ORIGENE-GenDiscovery Biotechnology, Inc and the pLenti-PGRMC1 vector constructed by inserting PGRMC1 cDNA obtained from pCMV6-PGRMC1 into the empty pLenti-IRES-Neo viral vector. X-tremeGENE Transfection Reagent (Roche Diagnostics, Mannheim, Germany) was used according to the manufacturer's instructions to generate the lenti-mock and lenti-PGRMC1 vectors. The X-tremeGENE Transfection Reagent was diluted with the OPTI-MEM medium (Invitrogen), and plasmid DNAs (pLenti-IRES-Neo and pLenti-PGRMC1) were added to the diluted X-tremeGENE Reagent. The X-tremeGENE–DNA mixture was introduced directly into HEK-293T cells. The medium was replaced after 24 h, and the supernatant, which contained lentivirus cDNA (Lt-Mock and Lt- PGRMC1) was collected after 72 h and filtered through 0.45-μm-pore filters. Virus infection efficiency was tested before ECC-1 infection by immunoblotting. ECC-1 cells were transduced with lenti-mock and lenti-PGRMC1 at two MOI (multiplicity of infection) treatment levels in 2 mL of medium + polybrene (8 μg/mL) and incubated at 37 C, 5 % CO_2 for 48 h, following which the medium was replaced with fresh RPMI-1640 culture medium. The transduced ECC-1 cells were selected in RPMI-1640 medium containing 1,000 μg/mL G418 (AMRESCO) for 2 weeks.

Flow cytometry analysis for apoptosis detection and cell cycle analysis

For apoptosis detection, the percentage of apoptotic cells stained using the FITC Annexin V Apoptosis Detection kit I (BD Biosciences) was determined. Cells were trypsinized and gently washed three times in cold phosphate buffered saline (PBS), following which the cells were re-suspended at a density of 10^6 cells/mL in 1× Annexin V binding buffer. Cells (10^5) were then stained with 2.5 μL FITC-conjugated Annexin V and 2.5 μL propidium iodide (PI). After a 20-min incubation, cells were analyzed using the Accuri CFlow system (BD Biosciences) by collecting emission data from the FL1 and FL2 channels at 530 and 575 nm, respectively, for 10^4 events. For cell cycle analysis, the cells were collected as mentioned above and fixed in 1 mL 70 % ethanol overnight, followed by rehydration in 9 mL PBS for 10 min at room temperature. The cells were then washed twice in PBS, resuspended in a PI staining solution (50 μg/mL PI stain and 100 μg/mL RNase in PBS) at a density of 10^6 cells/mL, and incubated on ice in the dark for 30 min. Staining intensity was analyzed in 10^4 cell using the Accuri CFlow system (BD Biosciences) by collecting emission data from the FL2 channel at 575 nm. Data were further analyzed using CFlow Plus analysis software (BD Biosciences).

Migration and invasion assay

The Transwell assay was used to examine the effect of PGRMC1 on migration and invasion in MES-SA cells. Transwell cell culture inserts (PET membrane) with an 8.0-μm pore size (SPL LIFE SCIENCES, Gyeonggi-do, Korea) were coated or not coated with Matrigel™ (BD Biosciences) in FBS-free McCoy's 5a medium. Matrigel™ was added to ice-cold FBS-free McCoy's 5a medium in the ratio of 1:3, and 50 μL used to coat the upper chamber of the transwell inserts. Complete medium (1 mL) was added to the lower chamber of the transwell as an attractant for invading cells. Cells were trypsinized in two washes in PBS, resuspended in FBS-free McCoy's 5a medium at a density of 5×10^5 cells/mL, seeded in the upper chamber, and then incubated at 37 °C for 18 h. The noninvading cells remaining on the upper surface of the filter were removed by a cotton swab. The filters were washed three times in PBS, fixed in 4 % paraformaldehyde for 20 min, and stained with crystal violet for 30 min. Cells which had invaded were visible under the optical microscope and were displayed at a magnification of 100×.

Immunofluorescence staining

For immunofluorescence staining, PGRMC1-overexpressing MES-SA cells and the relative control MES-SA cells were plated on 12-mm coverslips overnight. The cells were then fixed with 4 % paraformaldehyde for 25 min, and the attached cells washed twice in PBS, permeabilized with 0.1 % Triton X-100 for 10 min, blocked with 5 % bovine

serum albumin (BSA) in PBS for 1 h, followed by incubation with PGRMC1 antibody (1:100) at 4 °C for 24 h. After three PBS washes, samples were incubated with the appropriate fluorescently labeled secondary antibodies (546 nm) diluted (1:100) in 2.5 % BSA/PBS. For the localization of filamentous actin, cells were incubated with 0.1 μg/mL Alexa 488–phalloidin (Sigma, St. Louis, MO) for 45 min at 37 °C and the nucleus stained with 4′,6-diamidino-2-phenylindole (DAPI; 1:10,000) for 1 min. The coverslips were then washed three times with PBS and at least twice with double distilled H_2O before being mounted with 4 μL antifade mounting reagent (Invitrogen) and dried in the dark at 4 °C. For image analysis, the cells were imaged using a Zeiss Axiovert 200 M fluorescence microscope (Carl Zeiss, Jena, Germany). All laser intensities used to detect the same immunostained markers were the same, and all laser intensities used for capturing images were not saturated. Images were exported as .tif files using Zeiss Axioversion 4.8 software and processed using Adobe Photoshop version 7.0 software (Adobe Systems, San Jose, CA).

Results

Development of doxorubicin-resistant uterine cancer lines

In this study, we grew doxorubicin-sensitive MES-SA cells in doxorubicin-free medium containing 10 % (v/v) FBS. The MES-SA-resistant cell lines, MES-SA/DxR-2 μM and MES-SA/DxR-8 μM, were continuously exposed to 0.2 and 0.8 μM doxorubicin, respectively, to retain the doxorubicin resistance phenotype (see "Materials and methods" section for details). The doxorubicin-resistant lines were cultured in a drug-free medium for 1 week before the experiments. The IC_{50} of doxorubicin for the MES-SA, MES-SA/DxR-2 μM, and MES-SA/DxR-8 μM cells were 0.21, 2.28, and 15.49 μM, respectively (Fig. 1a). The proliferative rate of the resistant MES-SA/DxR-2 μM and MES-SA/DxR-8 μM cells was higher than that of the sensitive MES-SA cells (Fig. 1b). In addition, based on their different doxorubicin resistance levels, the MES-SA/DxR-2 μM and MES-SA/DxR-8 μM cells showed upregulated P-gp, glutathione S transferase, and PGRMC1 levels and downregulated topoisomerase II levels (Fig. 1c), thereby demonstrating a significant difference in doxorubicin resistance among the MES-SA, MES-SA/DxR-2 μM, and MES-SA/DxR-8 μM cells. These distinct physiological and biochemical characteristics make these cell lines suitable chemotherapy-resistant cell models for doxorubicin resistance-related research.

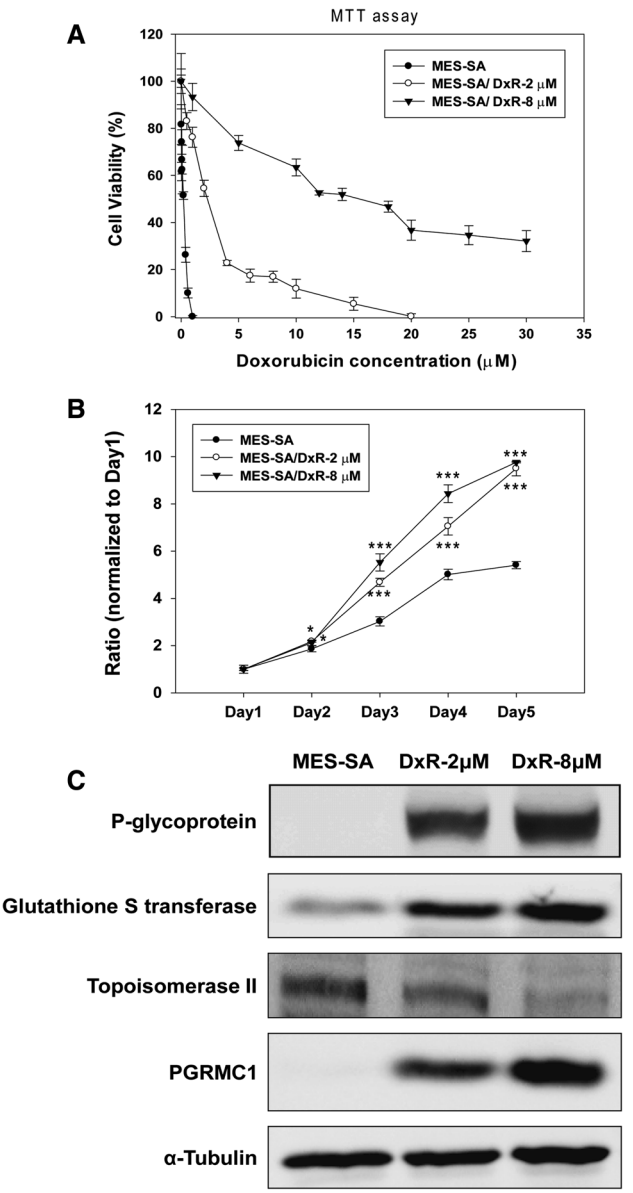


Fig. 1 Differences among the doxorubicin-sensitive MES-SA cells and the doxorubicin-resistant MES-SA/DxR-2 μM and MES-SA/DxR-8 μM cells in terms of viability, proliferation, and expression of reported drug resistance-related proteins. **a** MES-SA, MES-SA/DxR-2 μM, and MES-SA/DxR-8 μM cells were treated with doxorubicin in a dose-dependent manner for 48 h. **b** The growth rates of the MES-SA, MES-SA/DxR-2 μM, and MES-SA/DxR-8 μM cells were determined over 5 days. Absorbance values were normalized to the corresponding value oo day 1. Both cell viability and cell proliferation were determined using the MTT assay (see "MTT and CellTiter Blue Cell Viability Assay" section for details). **c** Expression of P-glycoprotein, glutathione S transferase, topoisomerase II, and progesterone receptor membrane component 1 (*PGRMC1*) in the MES-SA, MES-SA/DxR-2 μM, and MES-SA/DxR-8 μM cells was monitored by the immunoblotting assay. Data are expressed as the mean ± standard deviation (SD) of $n = 3$ measurements). $^{*}P < 0.05$; $^{***}P < 0.001$ vs. MES-SA

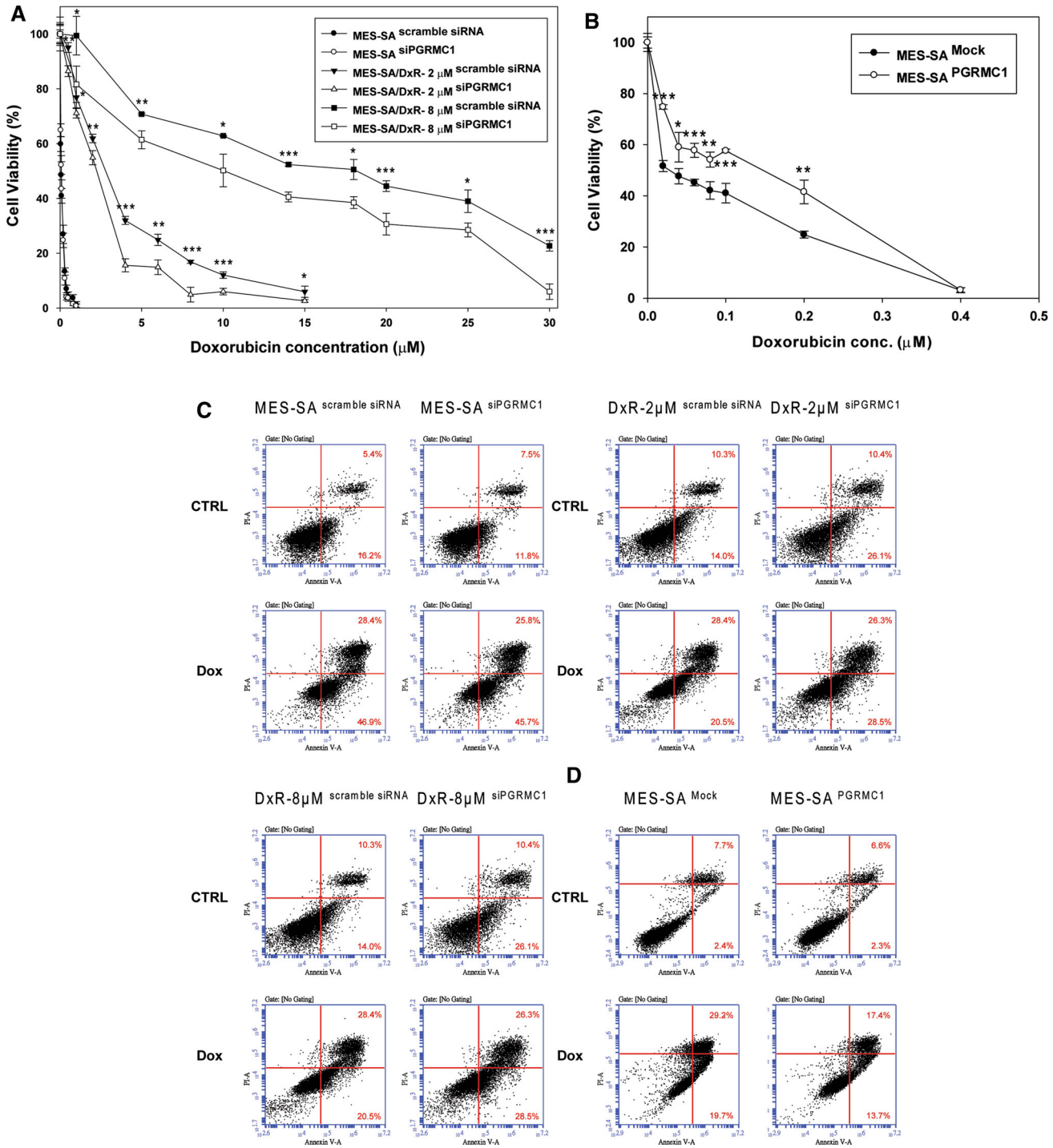
PGRMC1 decreases the sensitivity to doxorubicin and protects uterine sarcoma cells from apoptosis

In an earlier study, we found that PGRMC1 was overexpressed in resistant MES/SA-Dx5 cells and that PGRMC1 knockdown decreased resistant cell viability after doxorubicin treatment [10], implying that PGRMC1 might play a role in drug resistance. In the present study, upregulation of PGRMC1 expression depended on the drug resistance levels of the established resistant uterine sarcoma cell lines (Fig. 1c). To examine the role of PGRMC1 in drug resistance, we investigated siRNA-mediated PGRMC1 knockdown in the resistant uterine sarcoma cells, as well as PGRMC1 overexpression in the sensitive MES-SA cells. The efficiency of PGRMC1 knockdown and overexpression was examined using an immunoblotting assay, which revealed an up to 90 % reduction in PGRMC1 expression in the resistant MES-SA/DxR-2 μM and MES-SA/DxR-8 μM cells compared with the scrambled siRNA (mismatched siRNA sequence)-transfected control cells at 48 h after transfection [Electronic Supplementary Material (ESM) Fig. S1a]. In addition, the PGRMC1 cDNA-containing vector-transfected MES-SA cells stably overexpressed PGRMC1 compared with the empty vector-transfected MES-SA cells (ESM Fig. S1b). The 60-nM PGRMC1 siRNA-transfected MES-SA/DxR-2 μM and MES-SA/DxR-8 μM cells showed a significant attenuation of viability following treatment with the specified doxorubicin concentrations compared with the scrambled siRNA-transfected control cells. The IC_{50} of doxorubicin treatment against the scrambled siRNA-transfected MES-SA/DxR-2 μM and MES-SA/DxR-8 μM cells was 2.79 and 18.18 μM, respectively, whereas the IC_{50} for the PGRMC1 siRNA-transfected MES-SA/DxR-2 μM and MES-SA/DxR-8 μM cells was 2.25 and 10.08 μM, respectively. However, no significant difference was observed in MES-SA cell viability. The IC_{50} of doxorubicin treatment against both scrambled siRNA-transfected and PGRMC1 siRNA-transfected MES-SA cells was 0.08 μM (Fig. 2a); in contrast, PGRMC1 overexpression (IC_{50} 0.12 μM) dramatically increased the viability of the MES-SA cells after serial doxorubicin treatment compared with the empty vector-transfected control cells (IC_{50} 0.03 μM) (Fig. 2b). Further study based on the CellTiter Blue Cell Viability Assay showed that the trends of cell viability changes in PGRMC1 knockdown and overexpressing were consistent with the changes in MTT assay (ESM Fig. S2a, b).

We also used flow cytometry with PI staining and annexin V-conjugated Alexa Fluor 488 to analyze the percentages of apoptotic MES-SA/DxR-2 μM and MES-SA/DxR-8 μM cells induced by their IC_{50} of doxorubicin, with or without PGRMC1 knockdown. Briefly, early and late apoptotic cells, expressed as the percentage of apoptotic cells among the scrambled siRNA-transfected MES-SA/DxR-2 μM and MES-SA/DxR-8 μM cells, was 48.9 and 26.5 %, respectively, whereas that among the PGRMC1 siRNA-transfected cells was 54.8 and 44.1 %, respectively (Fig. 2c). In contrast, PGRMC1 knockdown had no direct effect on the doxorubicin-induced apoptotic population among the MES-SA cells. In addition, after 48 h treatment with doxorubicin at the IC_{50} of each cell line, the percentage of apoptotic cells among the PGRMC1-overexpressing MES-SA cells was significantly reduced (30.1 %) compared with that among the empty vector-transfected MES-SA cells (48.9 %) (Fig. 2d).

To further verify how PGRMC1 is involved in anti-apoptosis, we monitored the expression of apoptosis-related proteins in the PGRMC1-silenced MES-SA/DxR-8 μM cells, PGRMC1-overexpressing sensitive MES-SA cells, and their relative control cells. At 0.5 × IC_{50} of

Fig. 2 Effects of PGRMC1 knockdown and PGRMC1 overexpression on cell viability, apoptosis, and level of apoptotic factors in resistant (MES-SA/DxR-2 μM and MES-SA/DxR-8 μM) and sensitive (MES-SA) uterine sarcoma cells. **a** Effect of PGRMC1 knockdown on the viability of MES-SA, MES-SA/DxR-2 μM, and MES-SA/DxR-8 μM cells treated with doxorubicin in a dose-dependent manner. MTT-based viability assays were performed in which 7000 MES-SA, MES-SA/DxR-2 μM, and MES-SA/DxR-8 μM cells were seeded into 96-well plates for overnight incubation, followed by pretreatment with 60 nM PGRMC1-specific small interfering RNA (*siRNA*) or the corresponding GC content of scrambled (mis-matched) siRNA. Within 24 h, the cells were treated with the indicated doxorubicin concentrations for 48 h, followed by incubation with MTT for 4 h. Dimethyl sulfoxide (DMSO) was then added and the plates shaken for 20 min followed by absorbance measurement at 540 nm. Values were normalized against the untreated samples and are the average of four independent measurements ± SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. scrambled siRNA-transfected control cells. **b** Effect of PGRMC1 overexpression on the viability of the sensitive MES-SA cells. Values were normalized against the untreated samples and are the average of four independent measurements ± SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. empty vector-transfected control cells (*MES-SA* ^{Mock}). **c** Flow cytometry analysis of apoptosis using annexin V and propidium iodide (PI) staining. PGRMC1-silenced/scrambled siRNA-pretreated MES-SA, MES-SA/DxR-2 μM, and MES-SA/DxR-8 μM cells were treated with 1 × half-maximal inhibitory concentration (IC_{50}) of doxorubicin (*Dox*) or left untreated for 48 h (*CTRL*). After treatment, 10^6 cells were incubated with Alexa Fluor 488-conjugated annexin V and PI containing 1× binding buffer at room temperature for 15 min. The stained cells were analyzed by flow cytometry. **d** Effect of doxorubicin on apoptosis in the control MES-SA and PGRMC1-overexpressing MES-SA cells. Annexin V is presented on the *x-axis* as FL1-A, and PI is presented on the *y-axis* as FL2-A. The *lower right quadrant* indicates the percentage of early apoptotic cells (annexin V-positive cells), *upper right quadrant* indicates the percentage of late apoptotic cells (annexin V-positive and PI-positive cells). **e**, **f** Immunoblotting assay of indicated apoptotic factors with and without 0.5 × IC_{50} of doxorubicin for 24 h in the PGRMC1-silenced MES-SA/DxR-8 μM cells and PGRMC1-overexpressing MES-SA cells, respectively



doxorubicin, PGRMC1 knockdown induced p38 phosphorylation and downregulated Bcl2 (an anti-apoptotic factor) expression but upregulated Bax (a pro-apoptotic protein) expression, thus activating downstream caspase 9, caspase 3, and poly(ADP-ribose) polymerase (PARP) cleavage compared with the scrambled siRNA-transfected resistant MES-SA/DxR-8 μM cells (Fig. 2e). By contrast, compared with the mock control MES-SA cells, the PGRMC1-overexpressing MES-SA cells expressed lower amounts of phospho-p38 and showed no significant change in Bcl2 and Bax expression. In addition, PGRMC1-overexpressing MES-SA affected the downstream cleavage of caspase 9, caspase 3, and PARP after doxorubicin treatment (Fig. 2f). Thus, we conclude that PGRMC1 decreases

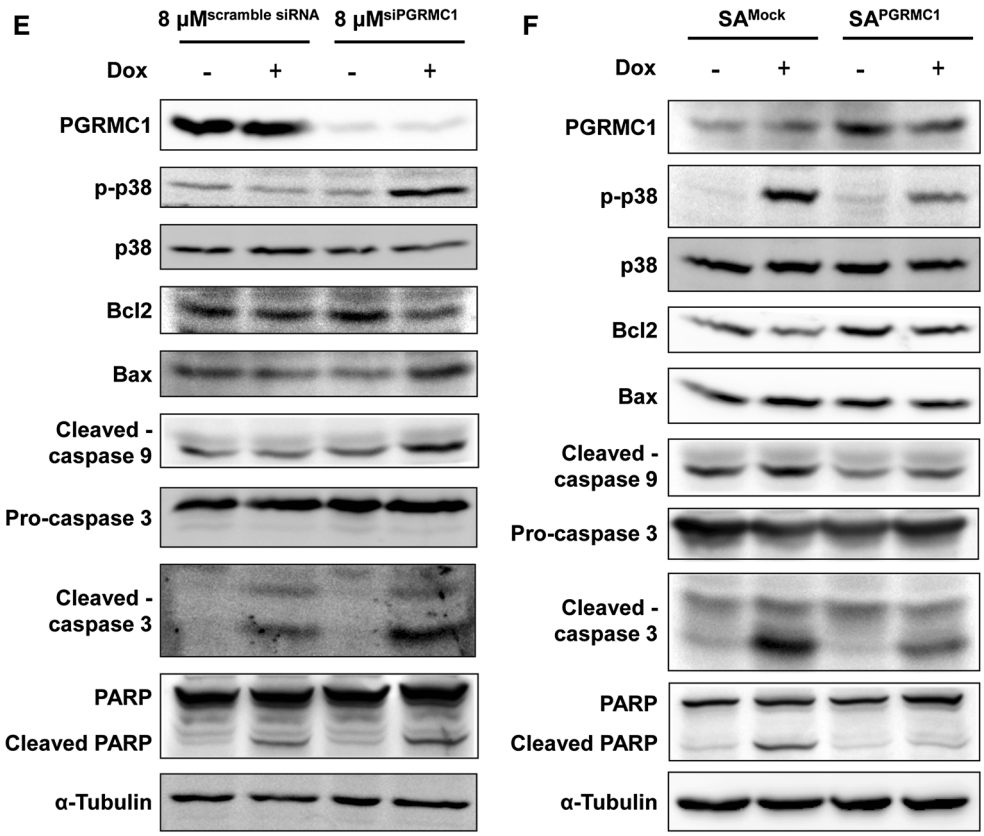


Fig. 2 continued

the sensitivity to doxorubicin and exhibits an anti-apoptotic effect in uterine sarcoma.

PGRMC1 promotes cell proliferation through phospho-ERK activation

To confirm the role of PGRMC1 in cell proliferation, we monitored the growth rate of PGRMC1-silenced MES-SA/DxR-8 μM, PGRMC1-forced MES-SA, and their relative control cells over 5 days; the ratios of cell proliferation were normalized to the absorbance of first seeded 3,000 cells. The proliferation rate of the PGRMC1–silenced MES-SA/DxR-2 μM and MES-SA/DxR-8 μM cells was significantly lower than that of the scrambled siRNA-transfected MES-SA/DxR-2 μM and MES-SA/DxR-8 μM cells (Fig. 3a). In addition, the PGRMC1-overexpressing MES-SA cells showed significantly enhanced cell growth during the 5-day period compared with the mock MES-SA cells, with up to a 1.6-fold difference ($P < 0.001$; Fig. 3b). The same trends were observed in the CellTiter Blue Cell Viability Assays (ESM Fig. S2c, d). To investigate the proliferation factor involved in PGRMC1-induced cell proliferation, proliferation-related MAPK and PI3K pathways were studied using the immunoblotting assay. Compared with the mock control cells, PGRMC1 downregulation in the MES-SA/DxR-8 μM cells reduced phospho-ERK expression and, in contrast, PGRMC1 upregulation in the MES-SA cells significantly induced phospho-ERK expression. However, no direct effect on Akt activation was observed in both the PGRMC1-silenced MES-SA/DxR-8 μM cells and the PGRMC1-overexpressing MES-SA cells compared with their relative control cells (Fig. 3c, d). Interestingly, phospho-ERK expression was downregulated in the empty vector-transfected MES-SA cells after doxorubicin treatment, whereas its expression was slightly reduced in the PGRMC1-overexpressing MES-SA cells treated with the same doxorubicin dose (ESM Fig. S4). These results suggest that PGRMC1 might improve cell proliferation through activation of the MAPK pathway.

PGRMC1 enhances cell cycle progression in uterine sarcoma cells

To examine whether PGRMC1 is involved in cell cycle progression, we used flow cytometry with PI staining to analyze the percentages of different cell cycle stages in the PGRMC1-silenced MES-SA/DxR-8 μM cells, scrambled siRNA-transfected MES-SA/DxR-8 μM cells, empty vector-transfected mock MES-SA cells, and PGRMC1-overexpressing MES-SA cells after incubation for 48 h in

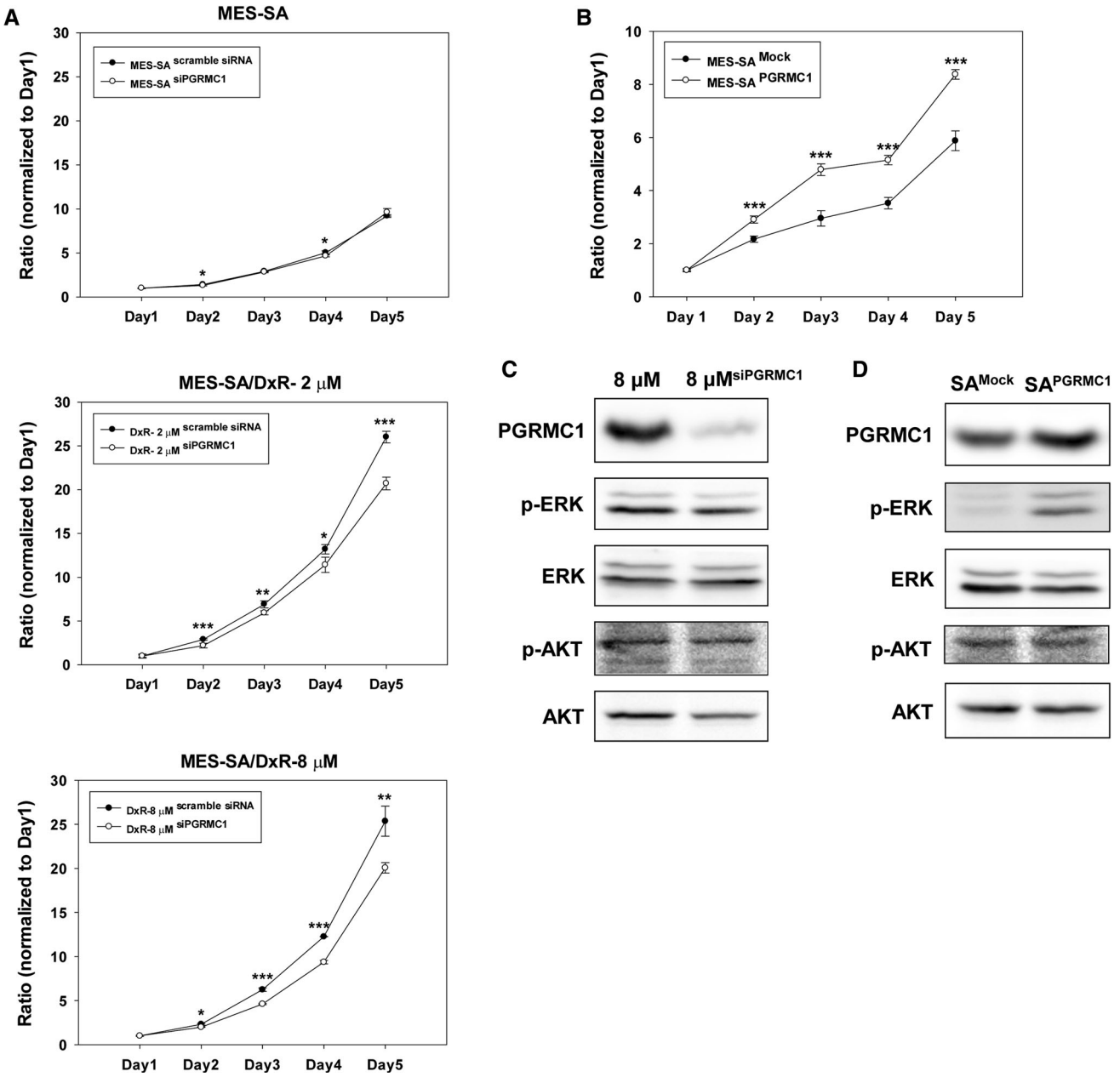


Fig. 3 Role of PGRMC1 in regulating uterine sarcoma cell proliferation. The growth rates of cells were determined by the MTT assay. **a** Effect of PGRMC1 knockdown on cell proliferation was evaluated from day 1 to day 5 in MES-SA, MES-SA/DxR-2 μM, and MES-SA/DxR-8 μM cells. Values were normalized against the corresponding average absorbance on day 1 and are the average of four independent measurements ± SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. scrambled siRNA-transfected control cells. **b** Effect of PGRMC1 overexpression on cell proliferation/growth in the sensitive MES-SA cells. **c**, **d** Immunoblotting assay of phospho-ERK and phospho-Akt was performed on 70 μg total proteins of the PGRMC1-silenced MES-SA/DxR-8 μM and PGRMC1-overexpressing MES-SA cells. *Blots* are representative of three independent experiments with similar results. Total ERK and Akt were used as the loading control

FBS-free medium. The percentages of the PGRMC1-silenced MES-SA/DxR-8 μM cells in the G0/G1 and S phases were 65.7 and 16.3 %, respectively, and those of the scrambled siRNA-transfected MES-SA/DxR-8 μM cells in these phases were 61.3 and 22.7 %, respectively (Fig. 4a). Compared with the mock control MES-SA cells, we observed an increase in the percentage of cells in the S phase in the PGRMC1-overexpressing MES-SA cells (from 12.0 to 21.1 %), with a corresponding decrease in the percentage of cells in the G1 phase (from 67.5 to 60.4 %) (Fig. 4b). Further, our analysis of crucial cell cycle regulatory proteins by the immunoblotting assay revealed that PGRMC1 knockdown downregulated cyclin E, cyclin A2, and FoxM1 expression and upregulated cyclin D2 and p27/

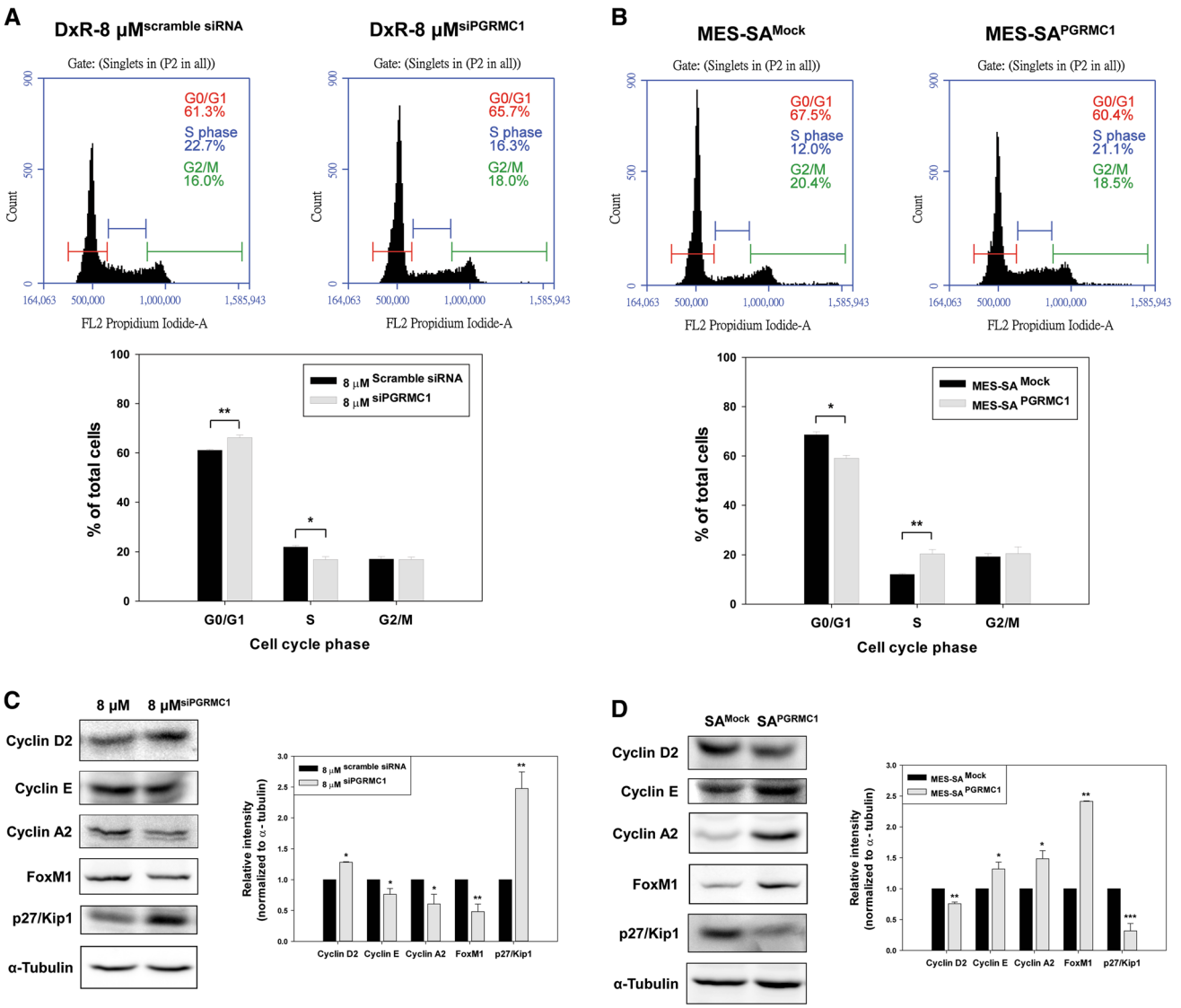


Fig. 4 Effect of PGRMC1 on cell cycle. Analysis samples were stained with PI and analyzed by flow cytometry. **a**, **b** Representative plots of cell cycle analysis of control, scrambled siRNA-transfected, PGRMC1-silenced MES-SA/DxR-8 μM, empty vector-transfected MES-SA, and PGRMC1-overexpressing MES-SA cells at 48 h. DNA content was measured by PI (*FL2-A*) staining on flow cytometry. The percentages of cell cycle phase (*G0/G1*, *S*, *G2/M*) are shown as indicated. Data are the mean of four independent measurements ± SD. The percentages of total cells in various stages of the cell cycle were quantified. $*P < 0.05$; $**P < 0.01$ vs. respective control cells. **c**, **d** Differential expression of the cell cycle regulatory proteins in response to PGRMC1 knockdown and overexpression. Immunoblotting assay was performed to compare the expression levels of cyclin D2, cyclin E, cyclin A2, FoxM1, and p27/Kip1 proteins among PGRMC1-silenced MES-SA/DxR-8 μM cells and scrambled siRNA-transfected control cells, both in the PGRMC1-overexpressing MES-SA cells and mock vector MES-SA cells. *Blots* are representative of three independent experiments with similar results. α-Tubulin expression was used as the loading control. The quantification of immunoblotting was conducted with Image Quant software. The values of the bands of the indicated proteins were normalized to α-tubulin. Expression ratios were normalized to expression in the respective control cells. Data are mean of three independent measurements ± SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. respective control cells

Kip1 expression compared to the expression of these proteins in the scrambled siRNA-transfected mock in the MES-SA/DxR-8 μM cells (Fig. 4c). Conversely, PGRMC1 overexpression increased cyclin E, cyclin A2, and FoxM1 expression levels but decreased cyclin D2 and p27/Kip1 expression levels in the MES-SA cells (Fig. 4d). Based on these results, we conclude that PGRMC1 overexpression resulted in the induction of the S and S/G2 phase regulators cyclin A2 and FoxM1 and the downregulation of in the G1 arrest inhibitor p27/Kip1. Thus, PGRMC1 may promote cell cycle progression to the S phase in uterine sarcoma cells.

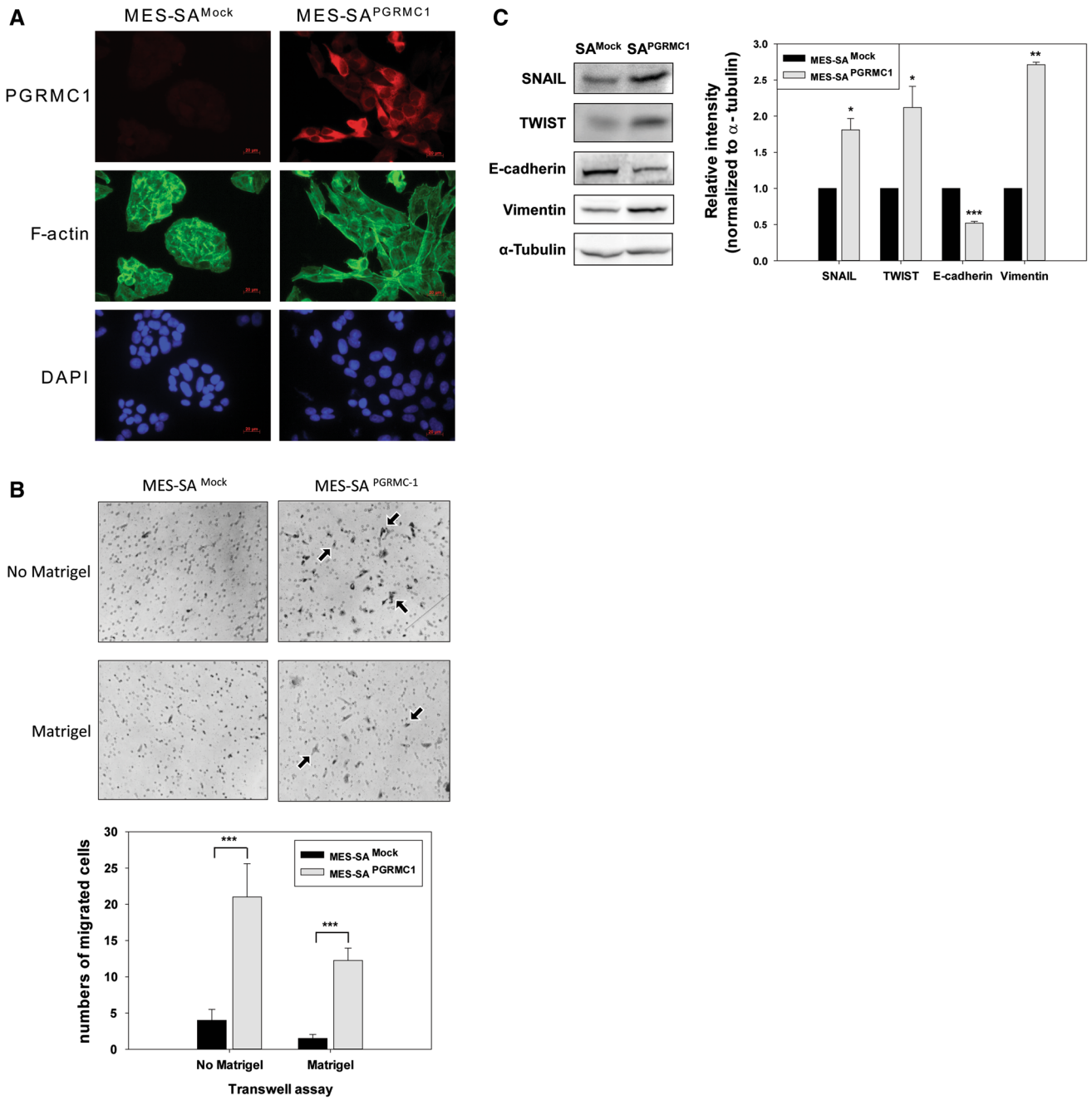


Fig. 5 PGRMC1 overexpression induced the epithelial–mesenchymal transition (EMT) phenotype in sensitive MES-SA cells. **a** Immunofluorescence representative image of morphological and protein organization changes in the PGRMC1 cDNA-containing vector- or empty vector-transfected MES-SA cells were seeded on cover slips for 24 h. The cells were fixed and stained for PGRMC1, F-actin, and 4′,6-diamidino-2-phenylindole (DAPI). Each set of three fields was taken using the same exposure, and images are representative of three fields. *Scale bar* 20 μm. **b** PGRMC1-overexpressing and empty vector-transfected control MES-SA cells were subjected to transwell cell migration assays and the Matrigel invasion assay. Representative images (×200) of migrated cells were stained with crystal violet (*arrows*) at 18 h after seeding on transwell chambers (8-μm pores). The number of crystal violet stained cells was calculated from six images of each condition (2 images of 3 independent experiments). Data are mean of six measurements ± SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. empty vector-transfected control cells. **c** The indicated proteins were detected in the MES-SA mock and PGRMC1-overexpressing MES-SA cells by the immunoblotting assay with α-tubulin as the loading control. Immunoblotting was quantified with Image Quant software. The values of the bands of the indicated proteins were normalized to α-tubulin. Expression ratios were normalized to the expression in empty vector transfected control cells. Data are the mean of three measurements ± SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. empty vector-transfected control cells

PGRMC1 overexpression enhances migration and invasion of doxorubicin-sensitive MES-SA cells

Previous studies have revealed that metastasis is associated with drug resistance [23, 24]. Figure 5a shows that there were increased levels of cytoskeleton F-actin reorganization and stress fiber formation in PGRMC1-overexpressing MES-SA than in the mock control MES-SA cells. The transwell assay was performed to analyze migration and invasive abilities between the mock control and PGRMC1-overexpressing MES-SA cells, with and without the Matrigel coating. The crystal violet-stained cell images shown in Fig. 5b demonstrate that PGRMC1 overexpression promoted the migration and invasion of the MES-SA cells . To further evaluate whether PGRMC1-induced metastasis occurred, we analyzed major epithelial–mesenchymal transition (EMT) indicator proteins, including SNAIL, TWIST, E-cadherin, and vimentin, by the immunoblotting assay. Compared with the protein expression level in the mock control MES-SA cells, PGRMC1-overexpressing MES-SA cells upregulated the expression of the transcription factors SNAIL and TWIST, thus downregulating E-cadherin expression and inducing vimentin expression (Fig. 5c). We therefore can conclude that PGRMC1 triggers EMT and promotes the migration and invasion of MES-SA cells.

Combination of PGRMC1 knockdown and verapamil significantly increases the sensitivity of resistant MES-SA/DxR-8 μM cells to doxorubicin

Verapamil is a calcium channel blocker, which has been used to cure angina and arrhythmias in clinical applications [25]. To assess P-gp overexpression in our established resistant uterine sarcoma cell lines (Fig. 1c), we evaluated the combination of siRNA-mediated PGRMC1 knockdown and verapamil to increase the sensitivity of the MES-SA/DxR-8 μM cells to doxorubicin. First, the effect of verapamil on the scrambled siRNA- and PGRMC1 siRNA-transfected MES-SA/DxR-8 μM cells was evaluated by the MTT assay. The PGRMC1-silenced MES-SA/DxR-8 μM cells were clearly more sensitive to verapamil than the scrambled siRNA-transfected control cells (Fig. 6a). The verapamil dose that maintained at least 80 % cell viability was chosen for the combination treatment of the MES-SA/DxR-8 μM cells. According to the results of the MTT and CellTiter Blue Cell Viability Assays, at a lower dose of doxorubicin, the viability of the PGRMC1-silenced MES-SA/DxR-8 μM cells was considerably lower than that of the scrambled siRNA-transfected control cells. In particular, PGRMC1 knockdown in 0.6 μM verapamil-treated MES-SA/DxR-8 μM cells synergistically induced the sensitivity of these cells to doxorubicin compared to scrambled siRNA transfection of the 0.6 μM verapamil-

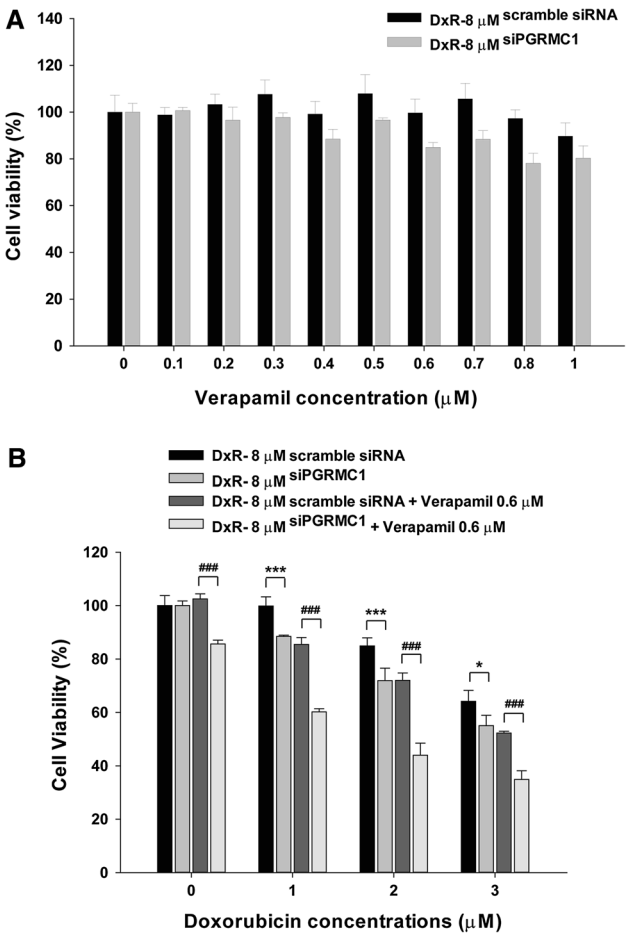


Fig. 6 PGRMC1 knockdown in combination with verapamil, a P-glycoprotein (P-gp) inhibitor, reduced the viability of resistant MES-SA/DxR-8 μM cells. **a** Effect of verapamil on the viability of scrambled siRNA-transfected control cells and PGRMC1-silenced MES-SA/DxR-8 μM cells. **b** Effect of PGRMC1 knockdown on the viability of the resistant MES-SA/DxR-8 μM cells, with and without 0.6 μM verapamil treatment, treated with doxorubicin in a dose-dependent manner. Values were normalized against the untreated samples of the scrambled siRNA-transfected control cells or PGRMC1-silenced MES-SA/DxR-8 μM cells. All values are expressed as the mean with a SD (*whiskers*) of three independent experiments. $^{*}P < 0.05$; $^{***}P < 0.001$ vs. scrambled siRNA-transfected control cells. With the 0.6 μM verapamil treatment, $^{\#\#\#}P < 0.001$ compared with the scrambled siRNA-transfected MES-SA/DxR-8 μM cells

treated control cells (Fig. 6b; ESM Fig. S2e), indicating that the combination of PGRMC1 knockdown and verapamil significantly suppressed MES-SA/DxR-8 μM cell viability to overcome multidrug resistance in uterine sarcoma.

Discussion

Chemotherapy not only serves as a major therapeutic strategy but also suppresses postsurgical tumor recurrence

in various cancers. Chemoresistance is a severe clinical problem in which the applied dose of the therapeutic drug reaches an uncontrollable level and decreases the effectiveness of the drug. The identification of novel therapeutic targets is essential for overcoming this obstacle to effective chemotherapy. As chemoresistance is an integrative cellular response, in an earlier study we used two dimension-difference gel electrophoresis (2D-DIGE) in combination with matrix-assisted laser desorption/ionization-time of flight tandem mass spectrometry (MALDI TOF/TOF MS) for the large-scale investigation of potent proteins involved in drug resistance mechanisms [10]. Among the proteins identified in that study, asparagine synthetase and PGRMC1 were further evaluated for their effects on cell viability to doxorubicin by the RNA interference (RNAi) method [10]. In present study, we used self-established resistant MES-SA/DxR-2 μM and MES-SA/DxR-8 μM and sensitive MES-SA uterine sarcoma cell lines as models to explore the role of PGRMC1 in drug resistance.

In the present study, increases in PGRMC1 expression were positively correlated to increased resistance levels in uterine sarcoma, suggesting the role of PGRMC1 in drug resistance. We elucidated the functional role of PGRMC1 by using knockdown and overexpression approaches. Hand et al. reported that Dap1p, a PGRMC1 homolog, is necessary for yeast survival after DNA damage [22]. PGRMC1 has also been found to be involved in P4-mediated anti-apoptosis due to cisplatin (a platinum agent)-induced cytotoxicity. PGRMC1 depletion induces apoptotic nuclei even after P4 treatment, which attenuates cisplatin-induced apoptosis in immortalized granulosa cells and ovarian cancer [18, 22, 26]. In our study, PGRMC1 decreased the sensitivity of cells to doxorubicin, which has a toxicity mechanism similar to that of cisplatin, and protected cancer cells from doxorubicin-induced apoptosis. In order to investigate the role of PGRMC1 in drug resistance across different types of uterine cancer, we chose the endometrial cancer ECC-1 cell line, which is a highly occurring type of uterine cancer, to overexpress PGRMC1. The cell viability and the proliferation results revealed that PGRMC1-overexpressing ECC-1 cells significantly increased both cell viability against doxorubicin and the proliferation rate in comparison to the control ECC-1 cells. These results imply that PGRMC1 might reduce drug sensitivity and promote cell proliferation in uterine cancer [ESM Fig. S4]. p38 activation has also been shown to be involved in doxorubicin-induced apoptosis [27, 28]. Our results suggest that PGRMC1 might attenuate p38 phosphorylation to inhibit doxorubicin-induced apoptosis.

Our data show that PGRMC1 promoted cell proliferation and cell cycle progression to the S phase by mediating ERK activation. These results are consistent with previous results in nonsmall cell lung cancer that siRNA-mediated PGRMC1 knockdown or AG-205 (an inhibitor)-mediated PGRMC1 inhibition downregulates ERK activation, thereby decreasing cell proliferation and G1 arrest in the cell cycle [17]. We further confirmed that the expression level of the cell cycle regulators was consistent with the effect of PGRMC1 on cell cycle progression. The CDK4/6-cyclin D complex in the early G1 phase is transferred to CDK2-cyclin E, leading to retinoblastoma hyperphosphorylation and complete E2F release [29]. Our results reveal that PGRMC1 increased cyclin E expression and decreased cyclin D expression, implying that the cells entered the next cell cycle stage. In addition, cyclin A overexpression in the S phase of fluorescence-activated cell sorting (FACS)-sorted cells [30, 31], was induced in the PGRMC1-overexpressing MES-SA cells and reduced in the PGRMC1-silenced MES-SA/DxR-8 μM cells. Furthermore, FoxM1 has been found to serve as a crucial regulator of both the G1/S phase transition and progression through the G2/M phase [32]. We therefore evaluated whether PGRMC1 upregulated FoxM1 expression. Interestingly, FoxM1 can modulate the G1/S phase transition by downregulating the CDK inhibitor p27/Kip1 [33]. In addition, we found p27/Kip1 to be decreased in the PGRMC1-overexpressing MES-SA cells. Thus, PGRMC1 may promote cell cycle progression by affecting these crucial cell cycle regulators.

It is a widely held belief among researchers, including the authors of this study, that the correlation between drug resistance and metastasis needs to be clarified [23, 24, 34, 35]. The EMT is the conversion of polarized nonmotile epithelial cells to nonpolarized motile mesenchymal cells through dissolution of the cell–cell barrier, such as the disolution of E-cadherin in metastasis. In a series of non-small cell lung cancers, Thomson et al. [36] reported that the higher expression level of E-cadherin, the epithelial cell junction protein, is more sensitive to erlotinib [an epidermal growth factor receptor (EGFR) inhibitor]. On the other hand, it has been elucidated that PGRMC-1 co-localizes with EGFR and improves susceptibility to erlotinib [37]. Therefore, the role of PGRMC1 in regulating drug resistance and metastasis must be further evaluated. Peluso et al. detected higher PGRMC1 levels in the metastatic stage of ovarian cancers [16], implying that PGRMC1 is associated with metastasis, and Ahmed et al. demonstrated that PGRMC1 knockdown decreases migration in lung adenocarcinoma [17]. The detailed PGRMC1-related metastasis mechanism must be further evaluated. Transforming growth factor-β, the EMT inducer, triggers changes in cytoskeletal reorganization and remodeling of cell–matrix connections [38]. Surprisingly, in our study, PGRMC1 overexpression dramatically changed the MES-SA cell pattern in F-actin remodeling and stress fiber formation, which implies that EMT might be induced by

PGRMC1. Of note, the EMT signaling mechanism elevates transcriptional repressors, such as the zinc finger protein SNAIL and the bHLH factor TWIST [39], to facilitate the subsequent transcriptional repression of E-cadherin and induction of vimentin expression. Changes in the expression of these aforementioned factors during EMT were also observed in the PGRMC1-overexpressing MES-SA cells.

The main reason for chemotherapy failure at the present time is drug resistance, which is an integrated and complicated response, including alteration of the drug target, enhancement of the survival pathway, and ineffective activation of cell death signaling. Rational chemotherapeutic agent combinations are usually proposed based on the synergy between drugs; however, targeting the same pathway may provide a relatively easy opportunity for cancer cells to escape. In their recent review, Holohan et al. highlighted that the targeting completely independent pathways—an approach referred to as orthogonal therapies—might be a more rational option to limit chemoresistance development [40]. Quan et al. studied endometrial stromal sarcoma (ESS), a type of uterine sarcoma, and found that histone deacetylase (HDAC) inhibitor suberanilohydroxamic acid (SAHA) in combination with the inhibitor of PI3K (LY294002, LY) and mTOR (rapamycin) could lead to synergistic growth inhibition and provide an optional therapeutic strategy for patients with ESS [41]. In our established resistant uterine cell lines, the immunoblotting assay revealed that P-gp was upregulated in response to the drug resistance level. P-gp belongs to the ATP-binding cassette transporter family, which is well known to efflux a wide range of structurally unrelated anticancer drugs [42], causing drug resistance in various cancers, such as those of the colon, kidney, pancreas, and liver. P-gp has been extensively investigated and its derived analogs further studied for their application in overcoming drug resistance. Verapamil, a calcium channel blocker, can directly bind to P-gp to reverse multidrug resistance through competition as a small molecule drug [43]. In our study, PGRMC1 depletion in combination with the nontoxic dose of verapamil significantly increased sensitivity to the low dose of doxorubicin in the highly resistant MES-SA/DxR-8 μM cells. Thus, it is possible that a combination of the drug efflux inhibitor verapamil and PGRMC1 could have a synergistic effect on doxorubicin cytotoxicity.

To conclude, we have demonstrated that PGRMC1 is a multifunctional protein providing resistance to cell pro-survival, anti-apoptosis, cell cycle progression, and EMT induction. Furthermore, targeting PGRMC1 in combination with the inhibitor of the different resistance pathways might be a preferred therapeutic option for overcoming tumor drug resistance.

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Conflict of interest None.

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