Oroxylin A exerts anticancer effects on human ovarian cancer cells via the PPARγ-dependent reversal of the progesterone receptor membrane component 1/2 expression profile

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Abstract. Ovarian cancer is the most lethal gynecological cancer worldwide. To date, the therapeutic approaches available for the treatment of ovarian cancer are still very limited. The present study first demonstrated that the Chinese herb, Oroxylin A, exerts inhibitory effects on both the migratory ability and viability of ovarian cancer cells. Notably, the inhibitory effects of the drug occurred in a dose-dependent manner. Oroxylin A only inhibited cell migration at the lower dose, whereas it induced early or late apoptosis at the middle or higher doses, respectively. Mechanistically, Oroxylin A increased peroxisome proliferator-activated receptor gamma (PPARγ) expression and altered the expression profile of progesterone receptor membrane component (PGRMC)1/2. Notably, PPARγ was revealed to play a central role in Oroxylin A-mediated anticancer activity. The silencing of PPARγ significantly abrogated Oroxylin A-induced apoptotic cell death and restored the expression profile of the PGRMC1/2 family in ovarian cancer cells. Collectively, the present study revealed that Oroxylin A exerted marked anticancer effects against ovarian cancer *in vitro*. Thus, Oroxylin A may have potential for use as a complementary therapy in the treatment of ovarian cancer.

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Introduction

Ovarian cancer is associated with the highest mortality rate among all types of gynecological cancers worldwide (1). The surgical removal of the tumor followed by platinum-based chemotherapy are the standard methods employed for the treatment of the disease (2,3). Despite these intense surgical and chemotherapeutic treatments, the recurrence of ovarian cancer is a frequent event (4). Reasonably, progesterone receptor membrane component (PGRMC)1, which is detected at relatively high levels in all ovarian tumors and cell lines and exhibits a high expression in the more advanced stages of ovarian cancer, is considered responsible for the development and chemoresistance of ovarian cancer (5,6). Mechanistically, PGRMC1 overexpression in ovarian cancer cells simultaneously promotes cell survival by enhancing epithelial growth factor receptor (EGFR) stabilization (7), and accelerates drug efflux by inducing cytochrome p450 activation (8). Moreover, PGRMC1 promotes substrate recycling to help tumor cell survival (9). In fact, PGRMC1 ligands have exhibited considerable potential in inhibiting tumor invasion and cancer progression in animal models of cancer (10). Thus, molecules with the capacity of PGRMC1 inhibition may prove to be beneficial for the treatment of ovarian cancer.

Flavonoids, the most abundant polyphenols in our daily diet, have been revealed to possess extensive pharmacological properties *in vivo* and *in vitro* (11). Oroxylin A is a flavonoid isolated from the root of *Scutellaria baicalensis* and displays multiple pharmacological activities, including anti-inflammatory, anti-viral, antioxidative and antitumor properties (12-14). Oroxylin A has been previously demonstrated as a competitive candidate of novel anticancer drugs in certain types of cancers e.g., breast cancer, glioma, hepatoma, leukemia and colorectal cancer, although not in ovarian cancer. The mechanisms underlying the anticancer effects of Oroxylin A vary, including

the induction of cell cycle arrest, the inhibition of metastasis, the induction of apoptosis, as well as other mechanisms (15,16). Furthermore, Oroxylin A modulates several signaling pathways, including nuclear factor-κB (17), hypoxia-inducible factor-1α/hedgehog (18), the ERK/GSK3β (19) pathways as well as numerous others. Although Oroxylin A has the potential to dynamically manipulate signaling pathways, the direct target of the molecule is relatively unknown. This was until Hui *et al* (20) observed an interaction between Oroxylin A and peroxisome proliferator-activated receptor gamma (PPARγ) in leukemia cells. PPARγ is a ligand-activated transcription factor of the nuclear receptor superfamily (21,22) and is related to immune response, inflammation and the pathogenesis of certain disorders, including obesity, atherosclerosis, and cancer (23). Recent studies have focused on the effects of PPARγ ligands functioning as anticancer agents. There has been a substantial accumulation of experimental data suggesting that PPARγ ligands induce the apoptosis of several types of cancer cells (24). As regards ovarian cancer, limited studies have suggested that PPARγ activation may inhibit the proliferation and induce the apoptosis of ovarian cancer cells by modulating multiple pathways (25,26). Oroxylin A has been revealed to function as an agonist of PPARγ successfully in leukemia cells, macrophages and endothelial cells (20,27,28); however, whether it can also activate PPARγ and induce inhibitory effects on ovarian cancer cells remains to be determined.

Since the role of PGRMC1 and PPARγ in cell survival and death in ovarian cancer is not yet fully known, and there is an urgent need for the development of novel alternative drugs for the increasing chemoresistance of the tumor, the present study focused on the effects of Oroxylin A on the proliferation, migration and apoptosis of ovarian cancer cells. In addition, the detailed mechanisms involved, including PPARγ and the PGRMC1/2 family were thoroughly investigated.

Materials and methods

Cells, transfection and reagents. The SKOV-3 ovarian cancer cell line and immortalized normal ovarian surface epithelial cell line IOSE80 were purchased from the American Type Culture Collection (ATCC). SKOV-3 or IOSE80 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen; Thermo Fisher Scientific, Inc.) with 10% (v/v) fetal calf serum (FCS; Gibco; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 100 ng/ml streptomycin (PAA Laboratories GmbH) in a 37˚C, 5% CO_2 humidified atmosphere. Small interfering RNAs for PPARγ knockdown were purchased from Shanghai GenePharma Co., Ltd. and an optimized siRNA for PPARγ was determined by immunoblotting. The sequences of a scrambled siRNA were 5'-ACGCGUAAC GCGGGAAUUUdTdT-3' (sense) and 5'-AAAUUCCCGCGU UACGCGUdTdT-3' (antisense), and that of PPARγ siRNA were: 5'-UCCAUAAAGUCACCAAAAGGCdTdT-3' (sense) 5'-CUUUUGGUGACUUUAUGGAGCdTdT-3' (antisense). siRNA transfections into SKOV-3 cells were performed using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Oroxylin A (MW: 284.26, HPLC ≥98%) was a product of Sigma-Aldrich; Merck KGaA. Oroxylin A was dissolved in aqueous dimethyl sulfoxide (DMSO) and delivered to the cells in media containing this solvent at a final concentration of <0.1% (v/v).

MTT cell viability assay. The viability of the SKOV-3 or IOSE80 cells were determined by methyl thiazolyl tetrazolium (MTT) assays according to the standard protocol. Briefly, SKOV-3 or IOSE80 cells were plated at a density of 2,000 cells/well with 100 μl of medium in 96-well plates with increasing concentrations of Oroxylin A (0, 20, 50, 100, 200, 400 and 800 μM, dissolved in DMSO). Following incubation for 24, 48, 72 or 96 h, 15 μl MTT (1 μg/μl) reagent were added into the medium. A total of 200 μl DMSO was added to each well to dissolve the formazan product. The optical density (OD) value was examined by a microplate spectrophotometer (Bio-Rad) at the wavelength of 560 nm.

Wound healing assay. The SKOV-3 ovarian cancer cells at 75% confluency in 12-well plates supplemented with DMEM containing 0.5% FCS was scratched using a sterilized 10-μl pipette tip, washed and then subjected to the vehicle or Oroxylin A (20 μM), respectively. The migration of the SKOV-3 cells into the wound was imaged and assessed at 0, 24 and 48 h after treatment. The images were analyzed by Image Pro-Plus version 6.0 (Media Cybernetics, Inc.) software. The results are representative of three independent experiments.

Cell invasion assay. The invasion of the SKOV-3 cells through 8-μm pores was examined using a Matrigel-coated Transwell cell culture chamber (Corning Costar; Corning Inc.). The lower chamber was filled with DMEM containing 10% FCS. Cells in a total volume of 100 μl at a density of $2x10^5$ cells/ml were seeded onto the upper chamber with the culture containing Oroxylin A at a dosage of 20 μM. Chambers were incubated at 37˚C, 5% CO_2 humidified atmosphere for 24 or 48 h. The remaining cells on the top surface of the membrane were removed using a cotton swab followed by three washes with PBS. The cells that migrated to the bottom surface of the chamber were stained with 0.5% crystal violet at room temperature for 20 min, and quantified by counting 5 fields on the membrane under a 20X objective.

Cell apoptosis assay. The percentage of ovarian cancer cells actively undergoing apoptosis was determined by flow cytometry using an Annexin V/PI assay kit according to the manufacturer's instructions as previously described (29). Briefly, SKOV-3 cells were incubated with increasing concentrations of Oroxylin A (0, 20, 50, 100, 200 and 400 μM) for 48 h, and the cells were then digested with trypsin and harvested. After washing with PBS, the number of cells were counted. For each group, a total of $1x10^5$ cells were resuspended in binding buffer at a concentration of $1x10^6$ cells/ml. Subsequently, 10 μl Annexin V and 5 μl propidium iodide (PI) were added to the mixture, and the cells were incubated at room temperature for ≥15 min in the dark. Following incubation, the percentage of apoptotic cells was analyzed by flow cytometry (FACScan; BD Biosciences). For the PPARγ knockdown assay, SKOV-3 cells transfected with PPARγ siRNA or scramble siRNA were subjected to treatment with Oroxylin A (100 μM) for 24 h, and the cells were then harvested. This was followed by the treatments as aforementioned.

Quantitative PCR. Quantitative PCR was employed to characterize the expression of PPARγ, PGRMC1 and PGRMC2 in SKOV-3 cells. Total RNA was isolated from the SKOV-3 cells using the RNeasy Plus Mini kit (Qiagen, Inc.). Total RNA extracts were treated with DNase I (Invitrogen; Thermo Fisher Scientific, Inc.) prior to reverse transcription to avoid DNA contamination, which could lead to false-positive results. cDNA was synthesized by incubating 1 μg of RNA with oligo(dT) and Muloney murine leukemia virus reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc). Primers for the subsequent PCRs were as follows: PPARγ, 5'-TGGAGT TCA TGCTTGTGAAG-3' and reverse, 5'-GCATTATGAGAC ATCCCCAC-3' (168 bp); PGRMC1 forward, 5'-GACCAA AGGCCGCAAATTCT-3' and reverse, 5'-CAGTGCTTCCTT ATCCAGGCA-3' (106 bp); PGRMC2 forward, 5'-AGG GGA AGA ACCGTCAGA AT-3' and reverse, 5'-AAGCCC CAC CAGACATTACA-3' (283 bp); GAPDH forward, 5'-CACCCA CTCCTCCACCTT TG-3' and reverse, 5'-CCACCACCCTGT TGCTGTAG-3' (110 bp). The reaction times were as follows: 1 min at 94˚C then 35 cycles of 30 sec at 94˚C, 30 sec at 60˚C and 60 sec at 72˚C, and finally 10 min at 72˚C. Subsequently, the relative level of gene expression was expressed as the ratio of the target gene mean value to the geometric mean value of the reference gene ($2^{-\Delta\Delta Cq}$) (30).

Flow cytometric analysis. To detect the expression of PPARγ, PGRMC1 or PGRMC2 in ovarian cancer cells, $1x10^6$ cells were harvested and suspended in cold PBS. After fixing with 80% methanol (5 min) and permeabilizing with 0.1% PBS-Tween for 20 min, the cells were incubated with 1X PBS/10% normal goat serum/0.3 M glycine to block non-specific protein-protein interactions followed by PPARγ mAb (1:100 dilution; sc-166731), PGRMC1 mAb (1:100 dilution; sc-135720) or PGRMC2 mAb (1:50 dilution; sc-100904; Santa Cruz Biotechnology, Inc.) for 30 min at room temperature. The secondary antibody used was Alexa Fluor® 488 goat anti-mouse IgG (H+L) (product no. ab150117; Abcam, Inc.) at a dilution of 1:2,000 for 30 min at room temperature. The acquisition of data for <10,000 events were collected and analyzed with the FACScan flow cytometer (BD Biosciences).

Western blot analysis and antibodies. Western blot analysis was performed as previously described (29,31). Anti-PGRMC1 (1:1,000 dilution; cat. no. ab224056) rabbit polyclonal antibody and anti-PGRMC2 (1:1,000 dilution; cat. no. ab241302) rabbit polyclonal antibody were purchased from Abcam Inc. Anti-GAPDH (1:2,000 dilution; cat. no. sc-32233) mouse mAb, and horseradish peroxidase (HRP)-conjugated goat anti-mouse (1:4,000 dilution; cat. no. sc-2004) and anti-rabbit IgG (1:4,000 dilution; cat. no. sc-2004) were purchased from Santa Cruz Biotechnology, Inc. Anti-PPARγ (1:1,000 dilution; cat. no. 95128) mouse monoclonal antibody was purchased from Cell Signaling Technology, Inc. Primary antibodies were incubated at 4˚C over night, and secondary antibodies were incubated at room temperature for 1 h.

Statistical analysis. All experiments were carried out at least three times in triplicate. Numerical data were expressed as the means ± SD and they were analyzed using one-way ANOVA and a post-hoc Bonferroni test (α=0.05) to compare the means of all the groups. Two group comparisons were analyzed by a two-sided Student's t-test. P-values were calculated using SPSS 22.0 software (IBM Corp.) and a P-value <0.05 was considered to indicate a statistically significant difference.

Results

Oroxylin A inhibits the proliferation of human ovarian cancer cells. To determine the effects of Oroxylin A on the proliferation of human ovarian cancer cells, MTT assays with a series of drug concentrations were first carried out. Using the SKOV-3 human ovarian cancer cell line, it was observed that Oroxylin A dose-dependently inhibited the proliferation of these cells. As revealed in Fig. 1A, when the drug concentration was <20 μM, no significant inhibitory effects of Oroxylin A on cell proliferation were observed. However, when the drug concentration was >50 μM, proliferation of the SKOV-3 cells decreased significantly, compared to that of the vehicle-treated cells. Notably, when the concentration of the drug was increased to 400 μM, Oroxylin A almost entirely abrogated the proliferation of the ovarian cancer cell line. In addition, the 50% inhibitory concentration (IC_{50}) of Oroxylin A for the treatment of the SKOV-3 cells at the time-point of 48 h was also determined (Fig. 1C), which was calculated as 60.85 μM (95% CI, 47.72-77.60). Moreover, a time-dependent association of the drug with the ovarian cancer cells was also observed. As revealed in Fig. 1A, with a concentration of 200 μM, the inhibition rates of the SKOV-3 cells treated with Oroxylin A for 24, 48, 72 and 96 h were 44.39±6.35, 63.91±2.46, 70.97±1.40, and 72.53±2.95% respectively, indicating a cumulative effect of Oroxylin A on ovarian cancer cells with time. Oroxylin A has been revealed to possess an anticancer effect with lower toxicity to normal cells in some cancers (32,33), therefore, whether the drug exhibits toxicity to normal ovarian epithelial cells was determined. As revealed in Fig. 1B, Oroxylin A had no effect on the cell viability of IOSE80 cells when the dosage was not over 200 μM, at which concentration cell proliferation was significantly inhibited in SKOV-3 cells (Fig. 1A), suggesting the lower toxicity of the drug on normal ovarian cells. These results indicated that Oroxylin A inhibited the proliferation of and induced cytotoxicity to ovarian cancer cells.

Oroxylin A inhibits the migration of human ovarian cancer cells. As a hallmark of cell viability, the migration of ovarian cancer cells was also determined in this study. To avoid the influence of the killing effect by Oroxylin A on cell migration, the SKOV-3 cells were cultured with Oroxylin A at a concentration of 20 μM, where no killing effect was exhibited. Subsequently, a wound healing assay and a Transwell chamber assay were employed to determine the migration and invasion of the SKOV-3 cells, respectively. As revealed in Fig. 2A and B, the wound closure of the SKOV-3 cells exposed to Oroxylin A (20 μM) for 24 and 48 h was 15.67±6.45 and 19.63±2.45%, respectively, whereas that of the vehicle-exposed cells was 69.00±5.15 and 76.40±2.18%, respectively. Concordant results were also revealed in the Transwell chamber assay (Fig. 2C and D). The number of SKOV-3 cells in the lower chamber treated with Oroxylin A for 24 and 48 h was 8.33±2.52 and 6.03±3.00%, respectively, compared to that of

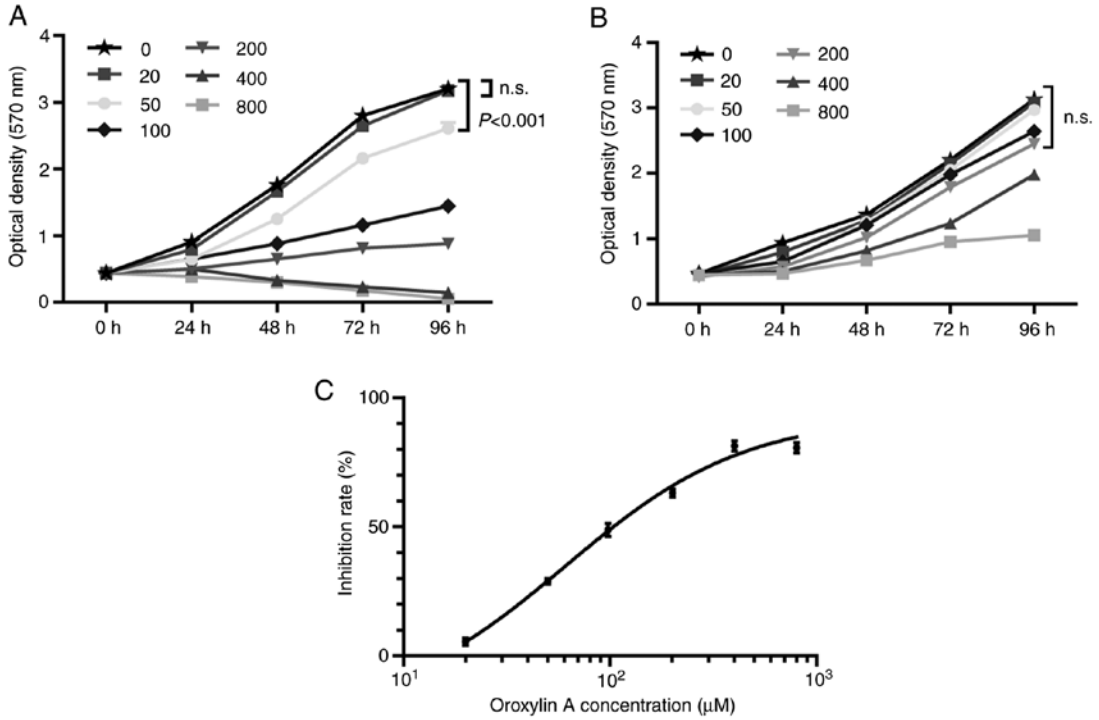

Figure 1. Oroxylin A inhibits the proliferation of SKOV-3 cells in a dose- and time-dependent manner. (A) MTT assay for the viability of SKOV-3 cells treated with vehicle or Oroxylin A (20, 50, 100, 200, 400 and 800 μM) for 24, 48, 72 or 96 h. (B) MTT assay for the viability of IOSE80 cells treated with vehicle or Oroxylin A. (C) IC_{50} of Oroxylin A on SKOV3 cells was conducted by logistic regression analysis.

the vehicle group which was 69.33±6.66 and 75.33±6.00%, respectively. These results revealed that Oroxylin A exerted a potent anti-migratory effect on ovarian cancer cells.

Oroxylin A induces the apoptosis of human ovarian cancer cells. Previous studies have revealed that Oroxylin A induces the apoptosis of various types of cells (15,34,35). Thus, an association between apoptosis and the inhibitory effect of Oroxylin A on ovarian cancer cells may exist. Annexin V/PI double-staining assay was then carried out to determine the effect of Oroxylin A on cell apoptosis. As revealed in Fig. 3A, the apoptosis of the SKOV-3 cells was induced by Oroxylin A in a dose-dependent manner. When the concentration was >50 μM, cell apoptosis was increased, with the results indicating that the cells had mainly undergone early apoptosis. However, along with the increasing drug concentration to 400 μM, the cells began to undergo late apoptosis. As revealed in Fig. 3B, the total apoptosis of the SKOV-3 cells exposed to Oroxylin A at concentrations of 20, 50, 100, 200, or 400 μM was 4.97±0.25, 22.08±4.99, 41.35±0.07, 60.20±0.85 and 58.35±7.28%, respectively. It is worth noting that the morphological alterations observed in Fig. 3C revealed that the cells exhibited cell shrinkage and chromatin condensation, also indicating the apoptosis of the SKOV-3 cells induced by Oroxylin A. In sum, the results of both flow cytometric assay and the morphological alterations of the cells indicated that Oroxylin A induced the apoptosis of human ovarian cancer cells.

PPAR gamma and PGRMC1/2 signaling are involved in Oroxylin A-mediated anticancer activity. PGRMC1/2 plays a vital role in ovarian cancer chemical resistance. PPARγ is a key factor modulating cancer metabolism and survival. Oroxylin A has been revealed as an agonist of PPARγ previously (20). The present study further detected the effects of Oroxylin A on the expression of PPARγ and the PGRMC1/2 family in ovarian cancer cells. As revealed in Fig. 4A, the mRNA levels of PPARγ and PGRMC2 were significantly increased in the SKOV-3 cells treated with Oroxylin A, compared to the cells treated with the vehicle. However, the mRNA level of PGRMC1 was markedly decreased following treatment with Oroxylin A. To validate the results of the mRNA levels, flow cytometry was then carried out to determine the protein expression. In line with the expression profiles of mRNAs, the protein levels of PPARγ and PGRMC2 were also revealed to be increased by Oroxylin A treatment, whereas PGRMC1 expression was also reduced (Fig. 4B and C). Concordant results were also manifested by western blot analysis with specific antibodies (Fig. 4D and E). These results indicated that treatment with Oroxylin A activated PPARγ and altered the expression profile of the PGRMC1/2 family in SKOV-3 cells.

PPARγ plays a central role in Oroxylin A-mediated anticancer activity. Considering the important role of PPARγ and the PGRMC1/2 family in ovarian cancer, whether PPARγ activation has an effect on PGRMC1/2 expression was determined. Subsequently, PPARγ knockdown assays were carried out (Fig. 1C). As revealed in Fig. 5A and B, PPARγ knockdown significantly attenuated the apoptosis of the SKOV-3 induced by Oroxylin A, compared to that of the scramble siRNA-treated SKOV-3 by Oroxylin A. These results indicated that the absence PPARγ attenuated Oroxylin A-induced cell apoptosis and that PPARγ plays an important role in Oroxylin A-mediated apoptotic cell death. Then the effects

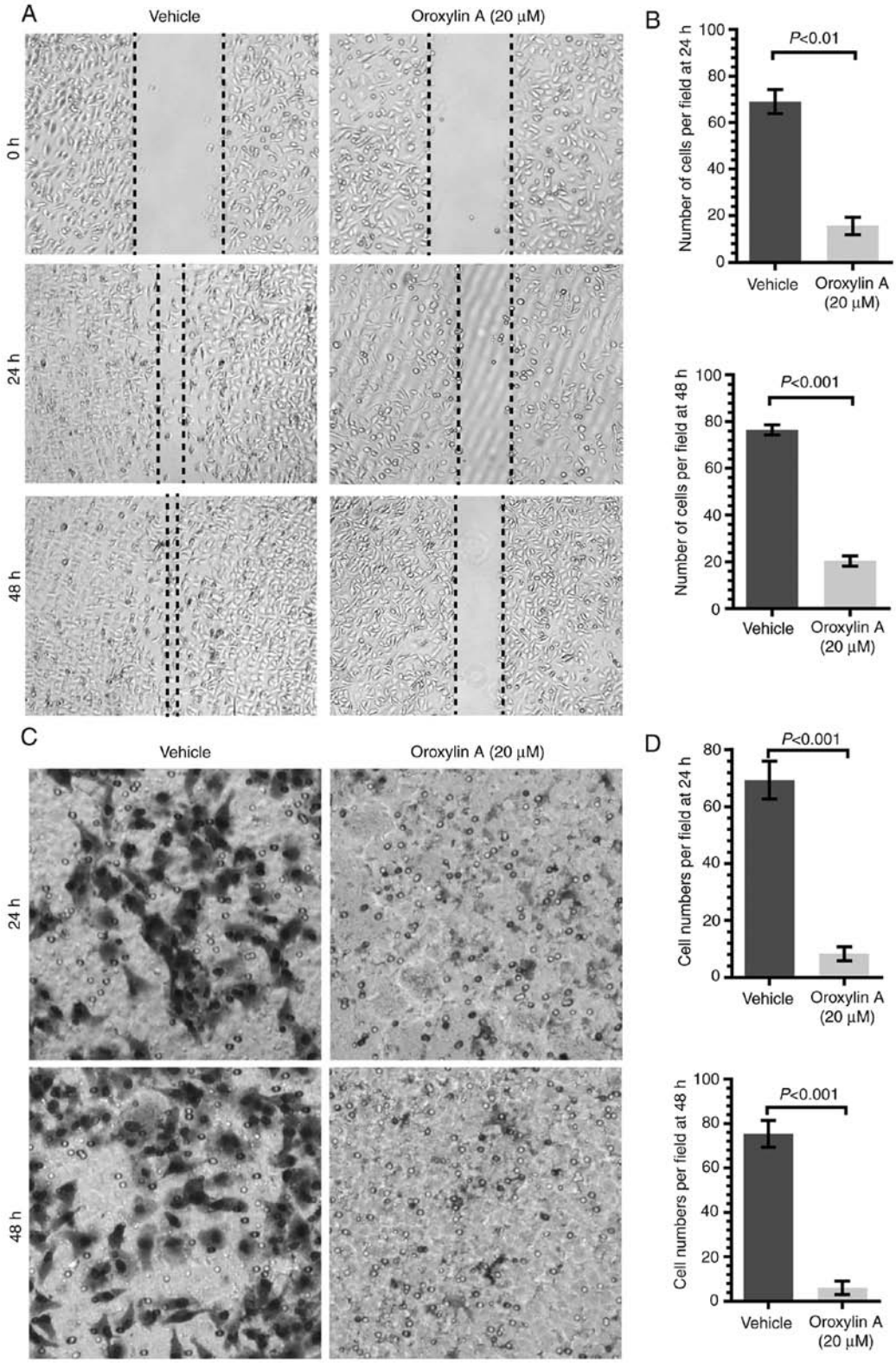

Figure 2. Oroxylin A inhibits the migration of SKOV-3 cells. (A) A monolayer of SKOV-3 cells was scratched with a sterile pipette tip. Cell migration of cells treated with or without Oroxylin A (20 μM) is presented. Cells were photographed at the initial wounding (0 h) and at 24 or 48 h post-wounding. (B) Bar graph at 24 or 48 h post-wounding represents the mean ± SE from three independent experiments. The areas of the lesions of the cells were quantified at the given time-points. (C) Images of cells invading through the porous membrane coated with Matrigel. (D) The average cell counts of invasive cells per 5 HPFs in three groups. The data represent the results obtained from at least three independent experiments. HPFs, high-power fields.

of PPARγ knockdown on the expression of the PGRMC1/2 family were further detected. As revealed in Fig. 5D, following treatment with Oroxylin A, PPARγ knockdown increased the expression of PGRMC1, while that of PGRMC2 was abolished, indicating a promising crosstalk between PPARγ and PGRMC1/2 pathways. Collectively, these results revealed that

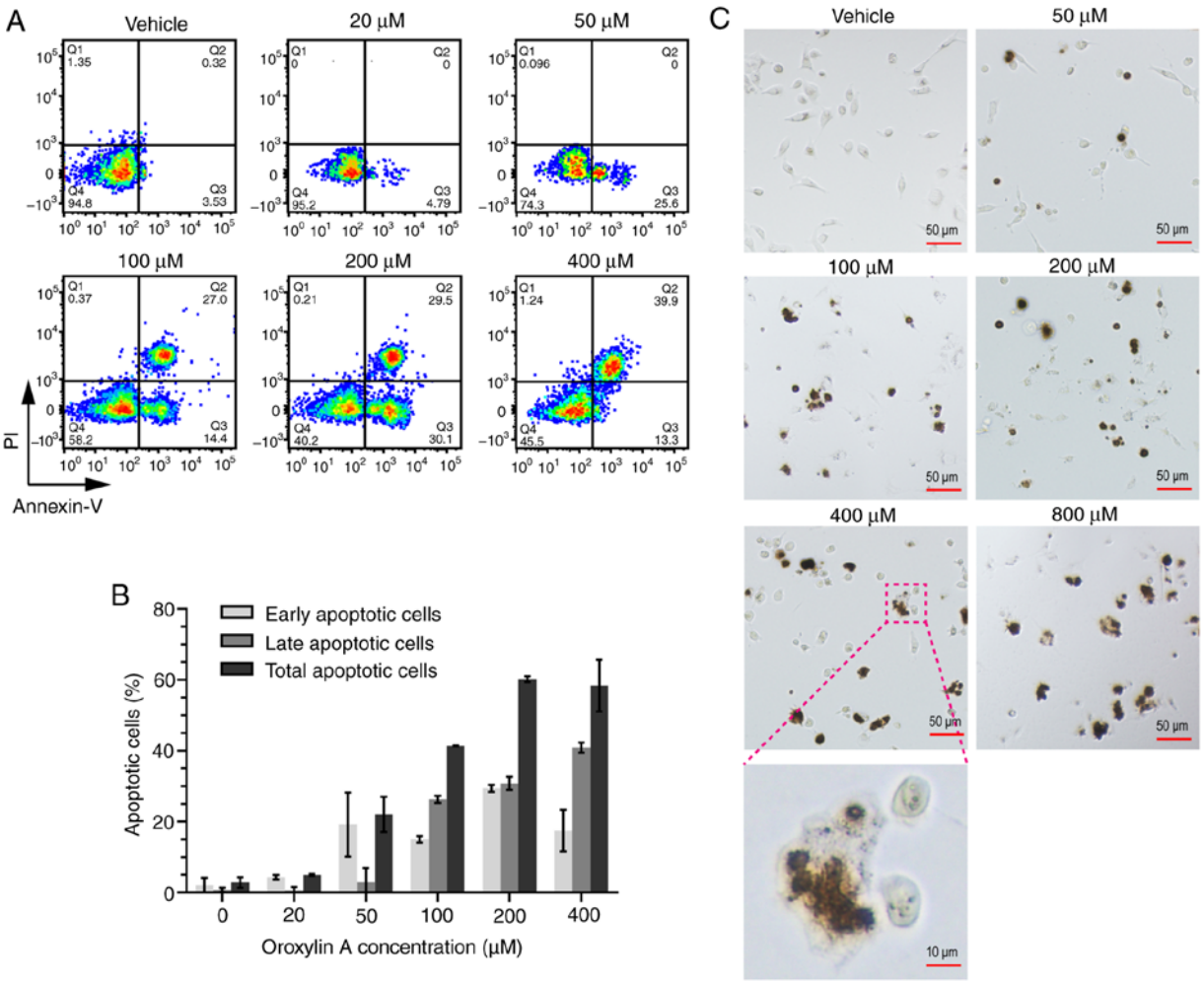

Figure 3. Oroxylin A induces the apoptosis of SKOV-3 cells. (A) Effect of Oroxylin A on the apoptosis of SKOV-3 cells. Cells were incubated with Oroxylin A at 0, 20, 50, 100, 200 and 400 μM, respectively. Apoptotic cells were harvested and examined at 48 h post-incubation. The x and y axes indicate Annexin V and propidium iodide (PI) staining intensities, respectively. (B) Summary data show the percentage of early, late or total apoptotic cells in panel A; the bars in the graph represent the mean ± SD; presented is one representative experiment out of three performed. (C) Morphological alteration of SKOV-3 cells treated with Oroxylin A compared with the vehicle- (negative control)-treated cells. Images of cells treated with Oroxylin A revealing cell shrinkage and chromatin condensation in SKOV-3 cells (original magnification, x100).

PPARγ activation mediated PGRMC1/2 expression and played a central role in the apoptosis of ovarian cancer cells induced by Oroxylin A.

Discussion

Oroxylin A is a novel anticancer drug used in China. To date, the anticancer effects of the drug have been determined in certain types of cancer, such as glioma (36), hepatocellular carcinoma (37), human gastric carcinoma (38), human breast cancer (39), human cervical cancer (40), colorectal adenocarcinoma (41), acute myelogenous leukemia (20), as well as many others. Although ovarian cancer has the highest mortality rate among all the types of cancers affecting women worldwide, to the best of our knowledge, there is no study available to date on the effects of Oroxylin A treatment on this disease. Herein, substantial experimental evidence was provided to indicate that Oroxylin A exerts a potent potential cell-killing effect on ovarian cancer cells *in vitro*. The present results indicated that Oroxylin A inhibited the proliferation of ovarian cancer cells in a dose- and time-dependent manner. Notably, even at a lower concentration of 20 μM, where no effect of the drug on cell proliferation was observed, Oroxylin A still inhibited the migration of the cancer cells robustly. Cell migration and invasion confer to cancer metastasis, which is the main cause of cancer recurrence. The anti-migratory effect of Oroxylin A against ovarian cancer cells at a lower concentration, suggests a promising extensive usage of the agent in the prevention and cure treatment of ovarian cancer patients.

In addition to its inhibitory effect on cell migration, Oroxlin A markedly inhibited cell proliferation by inducing apoptosis, which was more evident with the increasing concentration. Furthermore, when the drug concentration did not exceed 100 μM, early apoptotic cell death played a major role; however, when the concentration increased, late apoptotic cell death emerged and became dominant. The alteration of the apoptotic pattern with the increasing drug concentration suggests that there is an association between the apoptotic pattern and the drug concentration in Oroxylin A-treated ovarian cancer cells. PPARγ has been revealed to be a target of Oroxylin A in leukemia cells (20). Herein, it was also determined that Oroxylin A activated

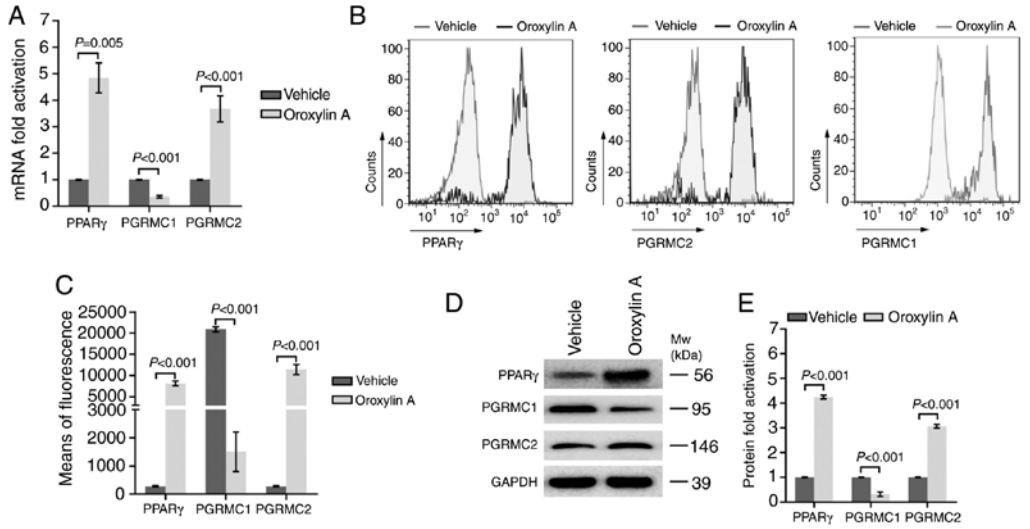

Figure 4. *PPARγ* and PGRMC1/2 signaling are involved in Oroxylin A-mediated anticancer activity. (A) mRNA expression profiles of *PPARγ* and *PGRMC1/2* in SKOV-3 cells treated with or without Oroxylin A. The relative levels of *PPARγ* and *PGRMC1/2* expressed as the ratio of the target gene mean value to the geometric mean value of *GAPDH*. (B) Representative flow cytometric histograms for *PPARγ* and PGRMC1/2 expression in SKOV-3 cells treated with Oroxylin A. Cells were stained with *PPARγ* or PGRMC1/2 mAb and fluorescence in isothiocyanate-labeled IgG was used as a secondary antibody. (C) Summary data revealed the fluorescence means of *PPARγ* and PGRMC1/2 in panel B; the bars in the graph represents the means ± SD; presented is one representative experiment out of three performed. (D) Representative immunoblot strips for PPARγ and PGRMC1/2 expression in SKOV-3 cells treated with Oroxylin A. (E) Summary data reveal the fold activation of PPARγ and PGRMC1/2 in panel D. *PPARγ*, peroxisome proliferator-activated receptor gamma; PGRMC, progesterone receptor membrane component.

PPARγ in the SKOV-3 ovarian cancer cells at both the mRNA and protein levels. This indicated that the activation of PPARγ by Oroxylin A is receptor-specific and is independent of the cell type. PPARγ is acknowledged as a suppressor in various types of cancer, and multiple chemical compounds exhibit anticancer activities by activating PPARγ (42-44). The inhibitory effect of Oroxylin A on ovarian cancer cells by PPARγ activation in the present study was in line with the findings of other studies on other types of cancer (43). In addition, PPARγ is necessary for the effects of Oroxylin A in treatment of the ovarian cancer cells. PPARγ silencing by specific siRNA significantly abrogated the apoptotic cell death of the ovarian cancer cells by Oroxylin A, indicating a central role of PPARγ in the effects of Oroxylin A.

The PGRMC1/2 family plays crucial role in the progression of ovarian cancer (6, 29). Both PGRMC1 and PGRMC2 belong to the membrane-associated progesterone receptor family, as they all contain a cytochrome b5-like heme/steroid binding domain. Currently, PGRMC1, which is highly expressed in ovarian cancer, has been extensively characterized as a tumor promoter by facilitating cancer proliferation and chemoresistance. PGRMC2, sharing an amino acid identity of ~89%, is strongly homologous to PGRMC1; contrarily, the limited available studies on PGRMC2 strongly imply a tumor suppressive function of the protein. The congenital contradiction of the two proteins indicates the key role of the PGRMC1/2 family in ovarian cancer progression. In the present study, it was first demonstrated that Oroxylin A reversed the expression profile of PGRMC1/2 in ovarian cancer cells by downregulating the expression of PGRMC1 and upregulating PGRMC2 expression. The present findings indicated that through the modulation of the expression profile of the PGRMC1/2 family, Oroxylin A may function as a candidate for the treatment of ovarian cancer.

Since Oroxylin A can influence both PPARγ signaling and the PGRMC1/2 family, whether a crosstalk exists between these two signaling pathways warrants investigation. Although there is no evidence to reveal the direct interaction between the two signaling pathways, notably, multiple signaling pathways, including the Wnt/β-catenin pathway (45,46) and NF-κB pathway (47,48) have been identified to be involved in modulating both PPARγ signaling and the PGRMC1/2 family. Considering the function of PPARγ as a transcription factor activated by Oroxylin A, it is possible that PPARγ may affect the expression of the PGRMC1/2 family, which is located on the cell membrane by modulating the aforementioned pathways. As anticipated, PPARγ silencing significantly restored Oroxylin A-induced PGRMC1 downregulation and PGRMC2 upregulation. However, whether the aforementioned pathways or the other mechanisms participate in the modulation of PGRMC1/2 expression by Oroxylin A warrants further investigation.

In conclusion, given the dual effect of Oroxylin A on both PPARγ elevation and PGRMC1/2 expression profile reversal, Oroxylin A inhibits the migration and induces apoptosis of ovarian cancer cells. Thus, Oroxylin A may have potential for use in the treatment of ovarian cancer.

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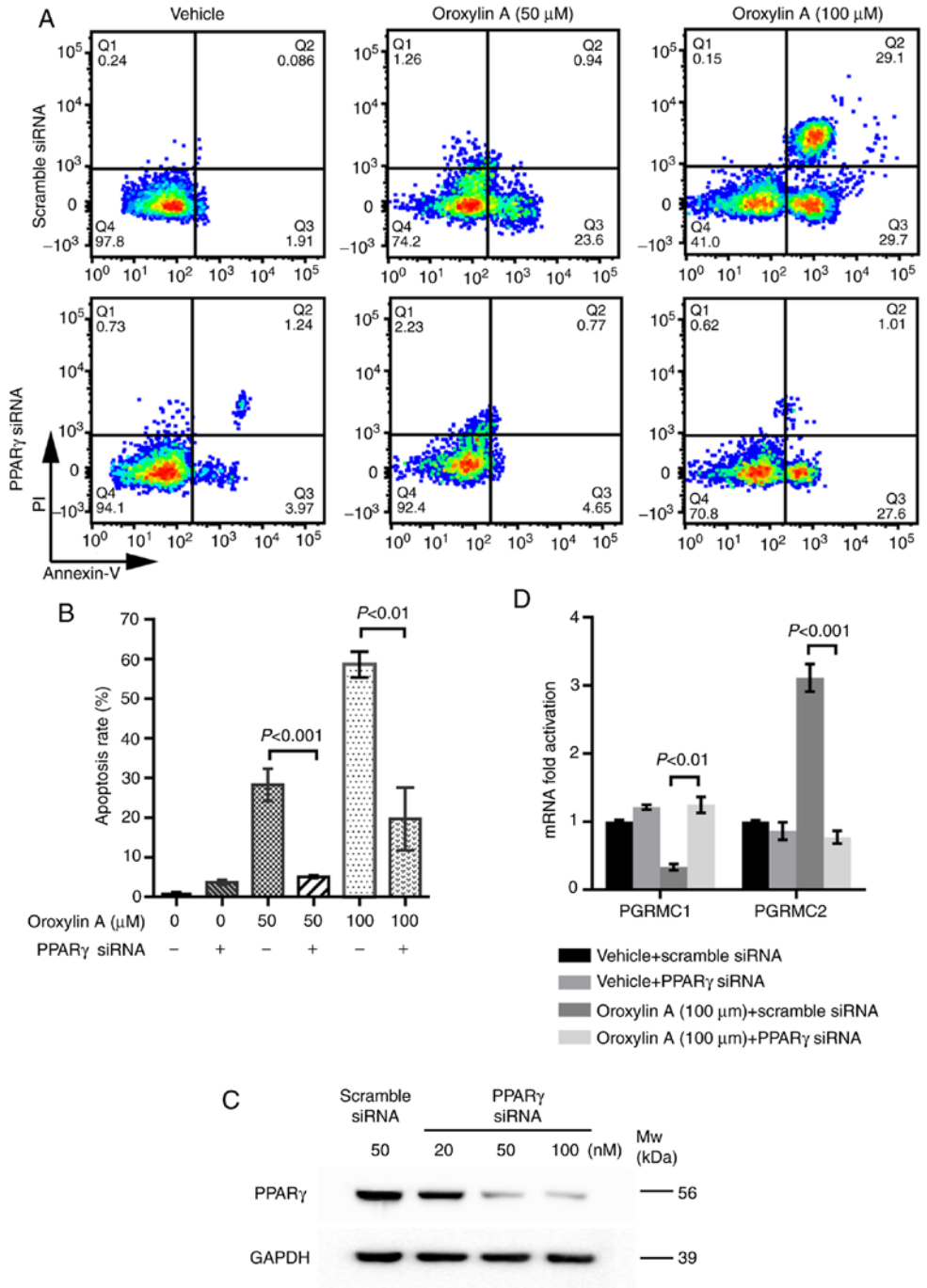

Figure 5. PPARγ plays a central role in Oroxylin A-mediated anticancer activity. (A) SKOV-3 cells transfected with or without siRNA against PPARγ for 24 h were treated with Oroxylin A (50 or 100 μM) and incubated for a further 24 h. Cells were harvested, and incubated with Annexin V and PI for apoptosis analysis. (B) Summary data reveal the percentage of total apoptotic cells in A. (C) An optimized concentration of PPARγ siRNA was determined by western blotting. (D) Relative mRNA expression profile of PGRMC1/2 in SKOV-3 cells treated as in A. *PPARγ*, peroxisome proliferator-activated receptor gamma; PI, propidium iodide; PGRMC, progesterone receptor membrane component.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JJS, YC and WJG conceived and designed the experiments. JJS, XFZ, JX and ZFW performed the experiments. YC and WJG analyzed the data. XFZ, JX and YC contributed the reagents/materials/analysis tools. JJS and YC wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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