

KDM5A promotes proliferation and EMT in ovarian cancer and closely correlates with PTX resistance

TONGFU FENG¹, YAN WANG², YAN LANG² and YUANZHEN ZHANG¹

¹Center of Reproductive Medicine, Zhongnan Hospital of Wuhan University, Wuhan, Hubei 430071; ²Department of Gynecology, Hubei Maternal and Children's Hospital, Wuhan, Hubei 430070, P.R. China

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Abstract. The authors initially performed reverse transcription-quantitative polymerase chain reaction to determine the expression profile of KDM5A in ovarian cancer tissues and adjacent normal tissue. Compared with adjacent normal tissue, it was identified that KDM5A was highly expressed in ovarian cancer tissues. Moreover, human ovarian cell lines also confirmed that KDM5A was highly expressed in ovarian cancer. KDM5A was especially highly expressed in SKOV3/paclitaxel (PTX) cells, which are resistant to PTX. Previous studies demonstrated that chemoresistance in cancer cells facilitates epithelial-to-mesenchymal transition (EMT). Following this, whether KDM5A influenced EMT and metastasis was investigated. The expression of KDM5A and N-cadherin were obviously higher in SKOV3/PTX cells than in SKOV3 cells. The expression of E-cadherin was decreased and the expression of N-cadherin was increased following ectopic expression of KDM5A, while the expression of E-cadherin was increased and the expression of N-cadherin was decreased following KDM5A depletion. Transwell and wound healing assays were used to explore the function of KDM5A in metastasis. The present results indicated that KDM5A facilitated EMT and metastasis in ovarian cells. Moreover, it was identified that P-glycoprotein was increased while KDM5A was expressed ectopically in SKOV3 cells. Following fluorescence-activated cell sorting flow cytometry analysis and CCK-8 assay all revealed that KDM5A regulated the PTX sensitivity in SKOV3 and SKOV3/PTX cells. In brief, KDM5A is a crucial oncogene that is significantly upregulated in ovarian cancer. Its expression is closely correlated with cancer cell proliferation, EMT and metastasis. KDM5A suppresses ovarian cancer cell apoptosis under PTX treatment.

Introduction

Ovarian cancer is the most common malignant carcinoma in the world (1). Currently, paclitaxel (PTX) is the most popular chemotherapy for ovarian carcinoma (2). But multidrug resistance (MDR) is a great problem of PTX treatment. There is a large percentage of patients that become resistant to PTX, causing relapse (3-5). Unfortunately, the mechanisms of MDR remain poorly understood. Many studies demonstrated that chemoresistance in cancer cells facilitated epithelial-to-mesenchymal transition (EMT) (6,7). EMT works as a crucial physiological process that serve a key function in cancer cell progression and metastasis (8). The characteristics of EMT are a loss of epithelial traits and a gain of mesenchymal traits. At the molecular level, several epithelial markers, for example α -catenin and E-cadherin, are significantly decreased, whereas mesenchymal markers, for example vimentin and N-cadherin, are obviously increased (9,10).

Histone modification is an important mechanism that regulates cell processes (11). KDM5A is a histone demethylase which is specific for H3K4 and it is required for cell development (12-14). Initially, KDM5A was identified as a retinoblastoma associated protein (15). In addition, the KDM5A homolog gene in *Drosophila* mutation cause differentiation and cell growth defects (16). Gene abnormal expression is closely correlated with human cancer development. Previously, there have been multiple reports revealing that KDM5A is highly expressed in gastric cancer (17), acute myeloid leukemia (18) and breast cancer (19).

However, the function of KDM5A in ovarian cancer is not quite clear. In the present study, the authors identified that KDM5A was highly expressed in ovarian cancer tissues and cell lines. In addition, KDM5A promoted EMT and metastasis in ovarian cell lines. Cell proliferation also regulated by KDM5A, but detailed mechanisms of how KDM5A regulates cell proliferation is still unknown. Moreover, the authors indicated that a high expression level of KDM5A was associated with PTX resistance. The above work indicated that KDM5A was a novel target of ovarian cancer.

Correspondence to: Dr Yuanzhen Zhang, Center of Reproductive Medicine, Zhongnan Hospital of Wuhan University, 169 East Lake Road, Wuchang, Wuhan, Hubei 430071, P.R. China
E-mail: yuanzhenzhangll@163.com

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Materials and methods

Ovarian tissue and cell lines. All ovarian patient tissue experiments were approved by Ethics Committee of the Wuhan

University (Wuhan, China). A total of 47 pairs of ovarian tissue samples and adjacent normal tissue samples were collected from 47 patients who were diagnosed with ovarian cancer.

Human normal ovarian cell line IOSE80 and ovarian cancer cell line SKOV3 and OVCA429 were purchased from American Type Culture Collection (Manassas, VA, USA). SKOV3/PTX, which is paclitaxel-resistant, was obtained from the Zhongnan Hospital of Wuhan University (Wuhan, China). All cells were cultivated in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 1% penicillin/streptomycin and 10% FBS at 37°C under 5% CO₂. In order to maintain the PTX resistance, SKOV3/PTX cells were treated with 2 nM PTX (Abcam, Cambridge, UK).

Cell transfection. Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to transfect cells. Overexpressed or silenced KDM5A in ovarian cancer cells was created using KDM5A or KDM5A small interfering (si)RNA respectively. Following 48 h transfection, cells were collection and subsequent experiments were performed. The siRNA sequences were as follows: KDM5A siRNA#1, 5'-AAGAGCUACAACAGGCUCGGU-3' (sense); KDM5A siRNA#2, 5'-AAGUCCUCUAGUAGUCUUGAA-3' (sense); scramble siRNA (SCR), 5'-UUCUCCGAAC-GUG UCACGUTT-3' (sense).

Reverse transcription-quantitative polymerase chain reaction analysis (RT-qPCR). RNA was extracted by TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), then synthesized cDNA through TransScript First-Strand cDNA Synthesis SuperMix (Transgen Biotech, Beijing, China). Relative mRNA expression was detected by SYBR-Green Mix (Roche Diagnostics, Basel, Switzerland). Primer pairs for cDNA amplification were as follows: 5'-ATGATCCCTGCTCTTCTG TG-3' (forward) and 5'-GATACCATCTTCCACAACCTT CAG-3' (reverse) for α -catenin; 5'-AATAAAGACCAAGTG ACCACC-3' (forward) and 5'-GCAGAATCAGAATTAGCA AAGC-3' (reverse) for E-cadherin; 5'-TCATTAATGAGG GCCTTAAAGC-3' (forward) and 5'-GTTTCAGGTAATCATA GTCCTGCT-3' (reverse) for N-cadherin; 5'-GTGAATACC AAGACCTGCTC-3' (forward) and 5'-ATCCAGATTAGT TTCCCTCAG-3' (reverse) for vimentin; 5'-GAACCATGA GAAGTATGACAACAG-3' (forward) and 5'-ATGGACTGT GGTCATGAGTC' (reverse) for GAPDH. GAPDH was used to as internal control. All experiments were performed three independent times.

Western blot analysis. Cells were initially lysed using radio-immunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and the protein concentration was quantified using the Bio-Rad protein assay kit (cat no. 5000001; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein (40 μ g) was separated using 12% SDS-PAGE gels and then transferred to a nitrocellulose filter membrane. The membrane was then blocked using skimmed milk and washed with PBS-1% Tween-20 solution. Membranes were incubated with indicated antibody at 4°C overnight and washed with PBS-1% Tween-20 solution. Then, membranes were incubated with secondary antibodies conjugated

with horseradish peroxidase (1:5,000; cat nos. A21010 and A21020; Abbkine Scientific Co., Ltd., Wuhan, China) at room temperature for 1 h. Protein bands were visualized using an enhanced chemiluminescence kit (Biorbyt, Ltd., Cambridge, UK). The primary antibodies were as follows: anti-KDM5A (1:2,500; cat no. SAB4301220; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany); anti-E-cadherin (1:2,000; cat no. ab1416); anti-N-cadherin (1:1,500; cat no. ab76057); anti- α -catenin (1:2,000; cat no. ab51032); anti-vimentin (1:3,000; cat no. ab92547); anti-P-glycoprotein (P-gp; 1:500; cat no. ab103477) (all from Abcam); and anti- β -actin (1:5,000; cat no. A1978; Sigma-Aldrich; Merck KGaA).

Cell Counting Kit (CCK)-8 assay. A CCK-8 assay was used to assess cell growth. In brief, cells were placed at a density of 5×10^3 cells/well in 6-well plates. A total of 10 μ l CCK-8 solution was added per 100 μ l medium and incubated 30 min at 37°C. Absorbance was detected at 450 nm. All experiments were performed three times.

Anchorage-independent growth assay. The method was conducted according to a previously described method (20). In brief, the culture dish was coated with 2X DMEM supplemented with 2% penicillin/streptomycin, 20% FBS and 1.2% Bacto agar (BD Biosciences, Franklin Lakes, NJ, USA) at a ratio of 1:1. Following ectopic expression of KDM5A in SKOV3 cells and after knocking down KDM5A in SKOV3/PTX cells, 1×10^4 cells were added to 2X DMEM medium supplemented with 2% penicillin/streptomycin, 20% FBS and 0.7% Bacto agar at a ratio of 1:1. Cells were incubated for 3 weeks, stained with 0.1% crystal violet at room temperature for 15 min and counted under a light microscope (magnification, $\times 20$). All experiments were performed three times independently.

Transwell assay. The Transwell chamber (Corning, Inc., Corning, NY, USA) was coated with Matrigel basement membrane matrix (BD Biosciences). Cells (density, 4×10^3) were added to the upper chamber and incubated with DMEM, which was serum-free, and the lower well contained 10% FBS. Cells were incubated at 37°C for 24 h. Cells were then fixed in methanol for 20 min, follow by staining with 0.1% crystal violet at room temperature for 15 min and washed with PBS solution. The number of invasive cells was counted under a light microscope (magnification, $\times 20$). All experiments were performed three independent times.

Wound healing assay. To detect cell migration, a wound healing assay was performed. In brief, 5×10^5 cells were placed in a 6-well plate and a pipette tip was used to scratch when the cells had reached 100% confluence. This was photographed immediately (time 0) and at 48 h. The ratio of cell migration into the scratch area was measured. All experiments were performed three independent times.

Drug sensitivity assay. Following ectopic expression of KDM5A in SKOV3 cells, and inhibition of KDM5A in SKOV3/PTX cells, 5×10^3 cells were plated in 96-well plates for 24 h at 37°C. Consequently, PTX was added at various concentrations (0, 5, 10, 20 and 40 nM) in DMEM medium. Following addition of PTX for 48 h, a CCK-8 assay was performed to

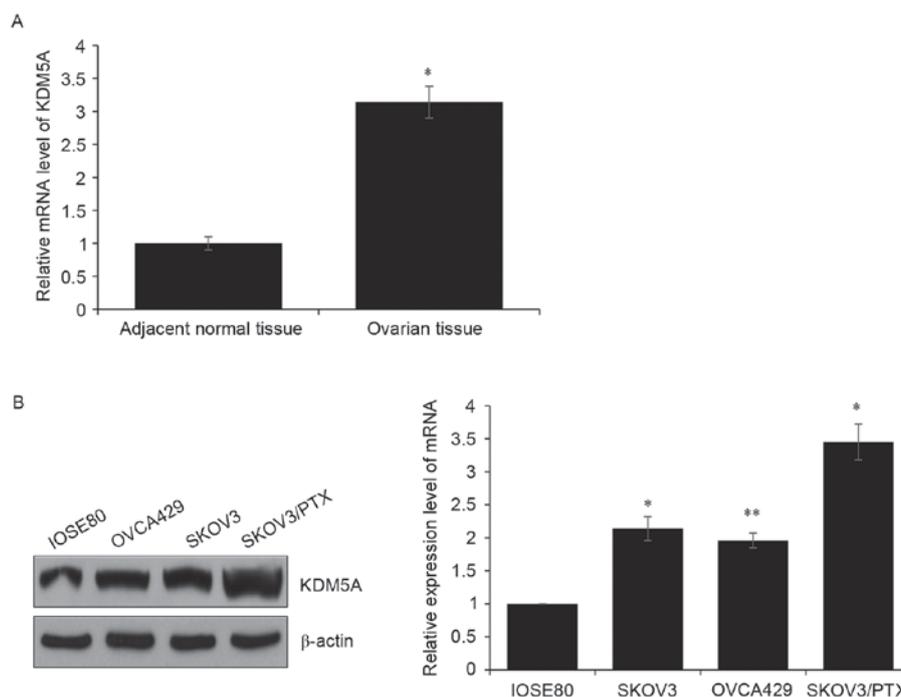


Figure 1. KDM5A is highly expressed in ovarian cancer and is associated with PTX resistance. (A) RT-qPCR analysis of KDM5A expression in ovarian tissue samples and adjacent normal tissue samples. The expression of KDM5A in adjacent normal tissue samples was used as a baseline to normalize the expression of KDM5A in ovarian tissue samples. * $P < 0.05$ vs. normal tissue. (B) Western blotting and RT-qPCR were used to determine the protein and mRNA level of KDM5A in human normal ovarian cell lines IOSE80 and human ovarian cancer cell lines, SKOV3, OVCA429 and SKOV3/PTX. * $P < 0.05$, ** $P < 0.01$ vs. IOSE80. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; PTX, paclitaxel.

assess cell viability. All experiments were performed three independent times.

Apoptosis assay. Following the ectopic expression of KDM5A in SKOV3 cells and the knocking down of KDM5A in SKOV3/PTX cells, 10 nM PTX was added for 48 h. Then, FACS flow cytometry was used to detect the percentage of apoptotic cells by Annexin V-FITC kit. All experiments were performed three independent times.

Statistical analysis. All data analysis was performed by SPSS software (version, 19.0; IBM PSS, Armonk, NY, USA). Expression of KDM5A in tissue samples or ovarian cell lines and human normal ovarian cell lines were analyzed by Chi-squared test. Student's t-test was performed to analyzing the results of metastasis, apoptosis assay and cell growth. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

KDM5A is highly expressed in ovarian cancer and is associated with PTX resistance. Aberrant gene expression contributes to tumor development. A previous report indicated that KDM5A is amplified in multiple tumors, including breast cancer (19). In order to investigate whether KDM5A serves a role in ovarian cancer, the authors collected 47 pairs of ovarian carcinoma tissues and adjacent normal tissues. Consequently, RT-qPCR was carried out to determine KDM5A expression level. It was observed that the KDM5A level in ovarian tissues was dramatically higher than that in normal

tissues (Fig. 1A). Meanwhile, KDM5A expression levels were measured in various ovarian cell lines (SKOV3, OVCA429 and SKOV3/PTX), compared with the human normal ovarian cell line IOSE80. The RT-qPCR and western blotting assays revealed both mRNA and protein levels of KDM5A were amplified in ovarian cell lines (Fig. 1B). Furthermore, expression of KDM5A was higher in SKOV3/PTX than other ovarian cell lines. These results indicated that KDM5A was associated with PTX resistance in ovarian cancer.

KDM5A facilitates EMT in ovarian cells. Numerous studies demonstrated that chemoresistance in cancer cells facilitated EMT (6,7). Detected EMT markers were identified in SKOV3 and SKOV3/PTX cells via western blotting. The expression of E-cadherin in SKOV3/PTX cells was lower than in SKOV3 cells, whereas the expression of N-cadherin in SKOV3/PTX cells was higher than in SKOV3 cells (Fig. 2A). Then, the authors further explored whether KDM5A regulated EMT in ovarian cancer. Two different KDM5A siRNAs were used to knockdown KDM5A in SKOV3 cells, and siRNA efficiency was detected by RT-qPCR and western blotting. As present in Fig. 2B, siKDM5A#1 and KDM5A#2 downregulated KDM5A ~80%, and siKDM5A#1 was more efficient, so siKDM5A#1 was used for further experiments (Fig. 2B). RT-qPCR and western blotting was again used to determine the EMT marker in SKOV3/PTX cells that were transfected with SCR or siKDM5A. The results indicated that, while there was a knockdown of KDM5A in SKOV3/PTX cells, E-cadherin and α -catenin were significantly increased. In addition, N-cadherin and vimentin were obviously decreased (Fig. 2C). Consequently, the authors expressed KDM5A ectopically in

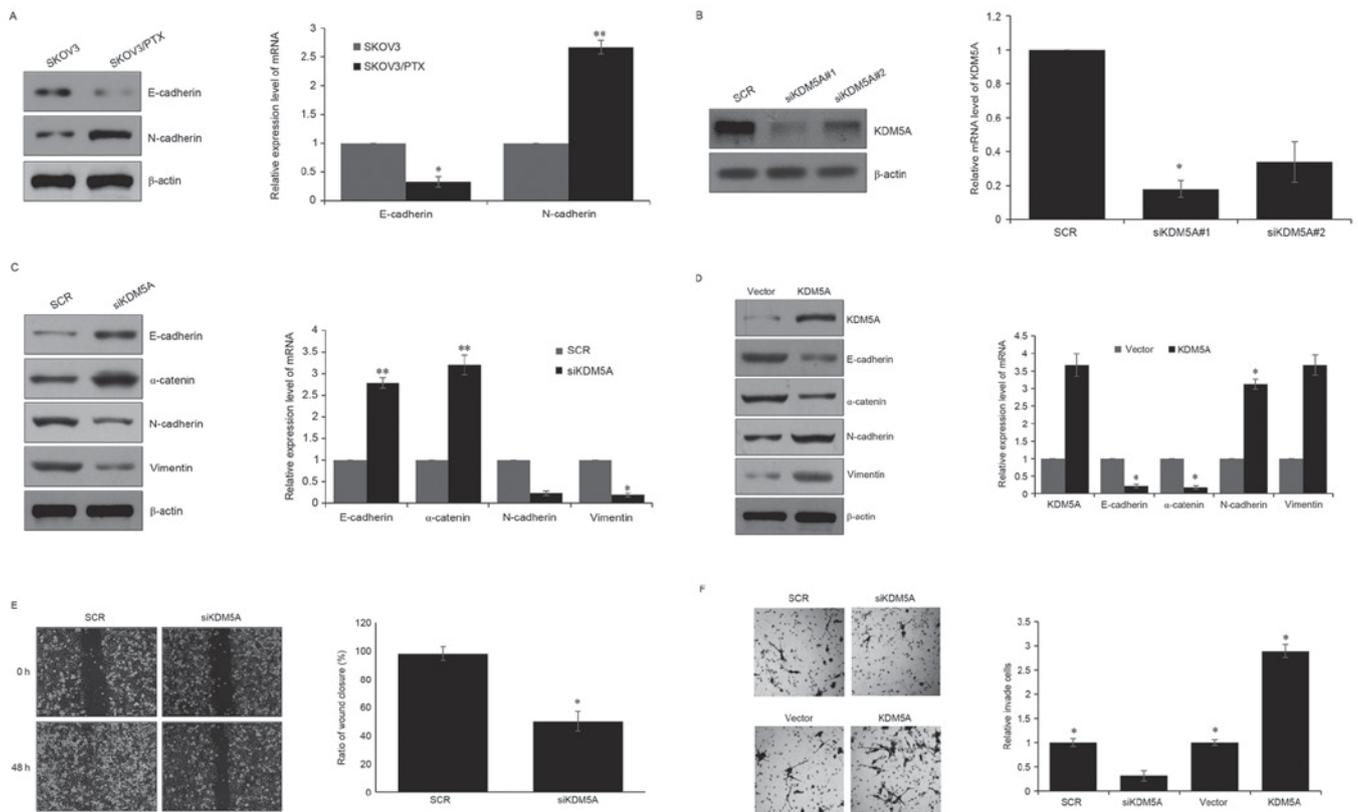


Figure 2. KDM5A facilitates the EMT in ovarian cells. (A) Western blotting and RT-qPCR were performed to determine the protein level and mRNA level of E-cadherin and N-cadherin in SKOV3 and SKOV3/PTX cells. (B) Two different KDM5A siRNA were transfected into SKOV3/PTX cells. Following 48 h transfection, western blotting and RT-qPCR were used to detect the efficiency of the siRNA. (C) KDM5A was then silenced in SKOV3/PTX cells, and, after a 48 h transfection, western blot and RT-qPCR were used to detect the protein level and mRNA level of EMT-associated protein. (D) KDM5A was expressed ectopically in SKOV3 cells, following 48 h transfection, western blotting and RT-qPCR were used to detect the protein level and mRNA level of EMT associated protein. (E) Wound healing assays were performed on SKOV3/PTX cells in which KDM5A was depleted to assess the migration ability of ovarian cells. The distance migrated was measured. All experiments were performed in three independent times. (F) Transwell assay was performed to determine the function of KDM5A in ovarian cells. All experiments were performed in three independent times. * $P < 0.05$, ** $P < 0.01$. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; EMT, epithelial-to-mesenchymal transition; PTX, paclitaxel; siRNA, small interfering RNA.

SKOV3 cells and detected the EMT markers. Results indicated that E-cadherin expression was decreased and N-cadherin expression was increased (Fig. 2D). These results suggested that KDM5A modulates the EMT in ovarian cells. EMT has been reported to promoted cancer cell metastasis, therefore wound healing and Transwell assays were performed to explore whether KDM5A regulated cancer cell metastasis. The wound healing assay revealed that the cells in which KDM5A was depleted migrated to ~50% of the wounded area, whereas the control groups migrated to ~100% of the area (Fig. 2E). In addition, the Transwell assay revealed that depletion of KDM5A obviously suppressed cancer cell metastasis, and ectopic expression of KDM5A significant facilitated cancer cell metastasis (Fig. 2F).

KDM5A modulates PTX sensitivity in ovarian cells.

Previously, KDM5A has been reported to serve an important role in erlotinib-resistant lung cancer (21). Moreover, KDM5A is more highly expressed in SKOV3/PTX cells than in other ovarian cells. Therefore, the authors assumed that KDM5A was also associated with PTX resistance in ovarian carcinoma. P-gp is a protein that is associated with drug transformation, and high expression of P-gp indicates drug resistance. The authors initially expressed

KDM5A ectopically in SKOV3 cells and silenced KDM5A in SKOV3/PTX cells. Following overexpression of KDM5A in SKOV3 cells, P-gp was increased, whereas when KDM5A was knocked down in SKOV3/PTX cells, P-gp was decreased (Fig. 3A). The CCK-8 assay was used to demonstrate the IC_{50} value of ovarian carcinoma cells. The results revealed that KDM5A suppression obviously increased the sensitivity of SKOV3/PTX cells to PTX, while ectopic expression of KDM5A decreased the sensitivity of SKOV3 cells to PTX (Fig. 3B). Consequently, FACS flow cytometry analysis was performed to investigate how KDM5A regulates cell apoptosis. The results demonstrated that overexpression of KDM5A decreased the percentage of apoptotic SKOV3 cells treated with PTX, while knocked down KDM5A obviously increased the percentage of apoptotic SKOV3/PTX cells treated with PTX (Fig. 3C).

KDM5A promotes proliferation of ovarian cancer cells.

To further assess the function of KDM5A on ovarian carcinoma transformation, KDM5A was knocked down in SKOV3 cells and a CCK-8 assay was performed to identify the role of KDM5A in cell growth. When KDM5A was depleted, cell growth was significantly inhibited (Fig. 4A). In contrast, when KDM5A was overexpressed in SKOV3 cells, the cell

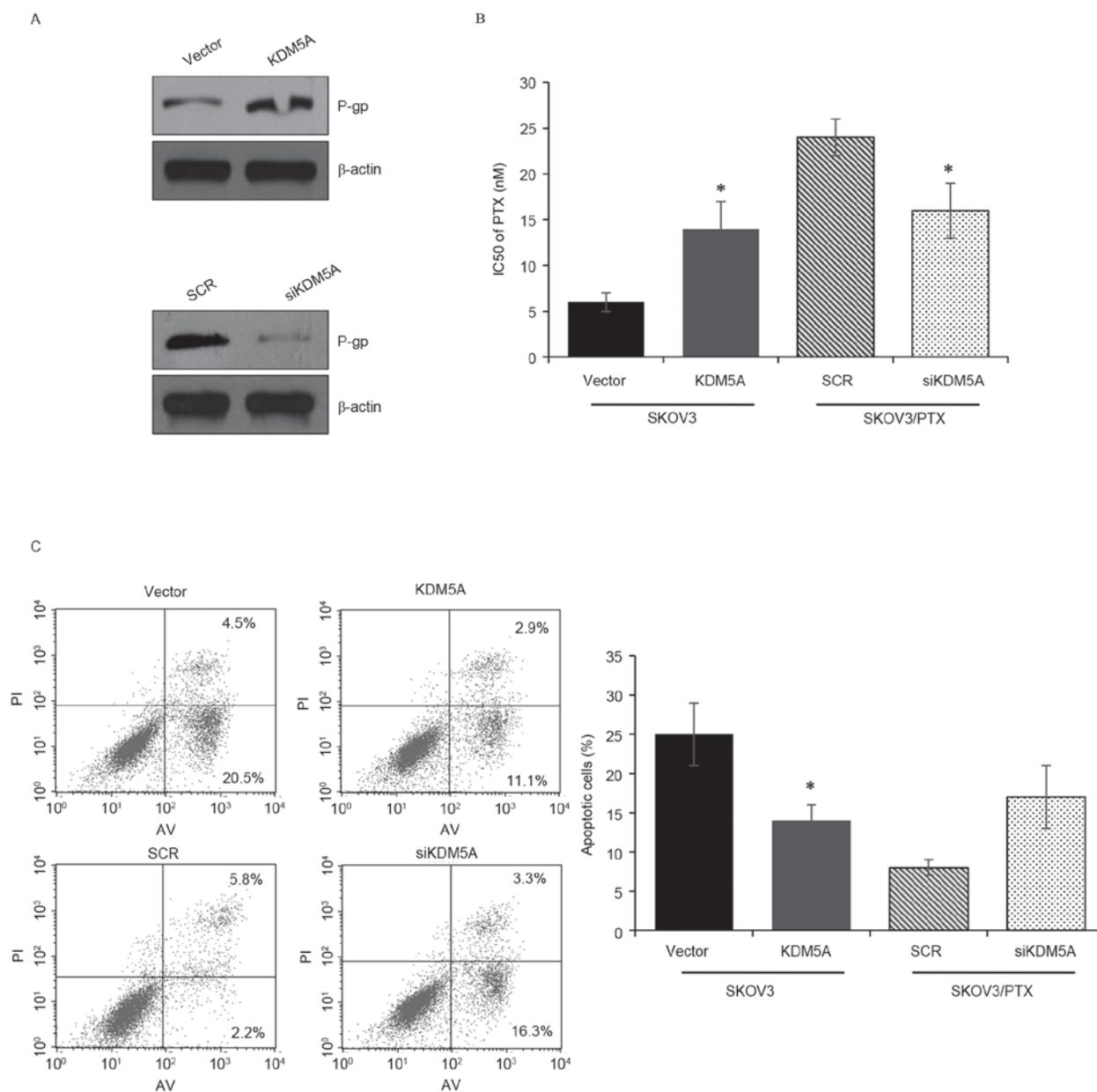


Figure 3. KDM5A modulates PTX sensitivity in ovarian cells. (A) Following 48 h transfection, P-gp expression was detected in SKOV3 cells with ectopic expression of KDM5A or with silenced KDM5A in SKOV3/PTX cells, through western blotting. (B) The same experiment was then conducted again, but instead to assess the PTX IC₅₀ value of SKOV3 or SKOV3/PTX cells through Cell Counting kit-8 assay. All experiments were performed three independent times. (C) FACS flow cytometric analysis was used to detect apoptotic cells stained with Annexin V-propidium iodide following treatment with 10 nM PTX for 48 h. All experiments were performed in three independent times. *P<0.05. PTX, paclitaxel; P-gp, P-glycoprotein; SCR, scramble small interfering RNA; siKDM5A, small interfering KDM5A.

growth was obviously increased (Fig. 4B). Furthermore, anchorage-independent growth assay also confirmed that overexpression of KDM5A facilitated cell anchorage-independent growth (Fig. 4C). Next, FACS flow cytometry was used to assess whether KDM5A regulated the cell cycle. When KDM5A was expressed ectopically in SKOV3 cells, the number of S phase cells was obviously increased (Fig. 4C). When KDM5A was silenced in SKOV3 cells, the number of S phase cells was obviously decreased and the number of G₀/G₁ phase cells was increased (Fig. 4D). The present study indicated that KDM5A facilitated cell proliferation via promoted cell into S phase.

Discussion

Ovarian cancer is the most common malignant carcinoma in the world, seriously threatening women's health (1). In the past decade, multiple chemotherapy drugs have emerged along with the development of cytoreductive surgery; however, the 5-year survival rate remains unsatisfactory. Drug resistance during chemotherapy is a crucial reason for the constant search for new drugs.

Some previous studies demonstrated that KDM5A serves a crucial role in drug-resistance in glioblastoma or breast cancer (19,22). Abnormal expression of KDM5A has been

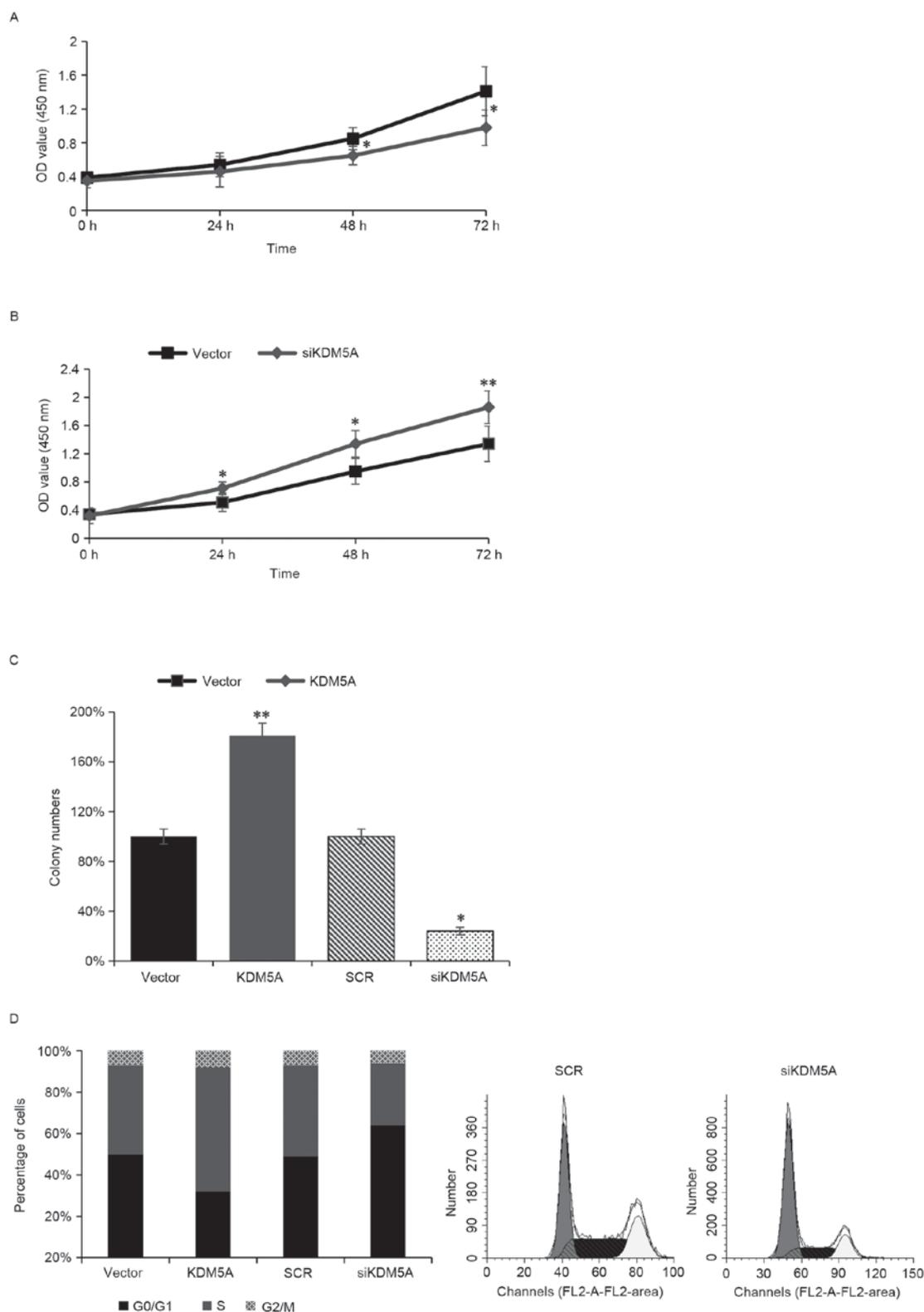


Figure 4. KDM5A promotes proliferation of ovarian cancer cells. (A) A CCK-8 assay was used to assess silenced KDM5A in SKOV3/PTX cells, at 48 h following transfection. All experiments were performed three independent times. * $P < 0.05$, ** $P < 0.01$ vs. 0 h. (B) A CCK-8 assay was used to assess silenced KDM5A in SKOV3 cells, at 48 h following transfection. All experiments were performed three independent times. * $P < 0.05$, ** $P < 0.01$ vs. 0 h. (C) An anchorage-independent growth assay was used to assess ectopically expressed or silenced KDM5A in SKOV3 cells, at 48 h following transfection. All experiments were performed three independent times. * $P < 0.05$, ** $P < 0.01$ vs. vector. (D) The function of KDM5A on the cell cycle was explored when expressed ectopically in SKOV3 cells or when silenced in SKOV3/PTX cells using flow cytometry. All experiments were performed three independent times. PTX, paclitaxel; siKDM5A, small interfering KDM5A; SCR, scramble siRNA.

reported in breast cancer (19) and glioblastoma (22). Although KDM5A regulated drug-resistance and other cell processes

in several cancers, the effect of KDM5A in ovarian cancer remains unknown, especially in PTX-resistant ovarian cancer.

Compared with normal ovarian tissues, the authors indicated that ovarian cancer tissues had an obviously high expression of KDM5A. As a result, the authors decided to further investigate its expression in ovarian cell lines. As expected, KDM5A was highly expressed in ovarian cell lines, compared with normal ovarian cell lines. Moreover, the expression level of KDM5A in SKOV3/PTX was higher than other ovarian cell lines that lack PTX resistance. The authors hypothesized that KDM5A effected PTX sensitivity in the ovarian cancer cells. Meanwhile, there are many previous studies demonstrated that chemoresistance in cancer cells facilitate EMT (6,7). The authors first investigated whether KDM5A regulates the EMT in ovarian cancer cells. The results revealed that overexpression of KDM5A promoted the EMT, epithelial marker levels were decreased, and the mesenchymal markers were increased. While KDM5A was silenced, the EMT was suppressed. The EMT has been indicated to facilitate cancer cell metastasis (23,24). The authors next detected whether KDM5A regulates ovarian cell metastasis. Transwell and wound healing assays confirmed that KDM5A served a crucial role in ovarian cell metastasis. The study's previous results indicated that KDM5A was expressed more highly in SKOV3/PTX cells than in other ovarian cell lines; therefore, the further investigated the function of KDM5A on PTX sensitivity. The expression of P-gp was increased following ectopic expression of KDM5A in SKOV3 cells, however the expression of P-gp was decreased following silencing KDM5A in SKOV3/PTX cells. FACS flow cytometry analysis and CCK-8 assay both confirmed that KDM5A inhibition facilitated the PTX sensitivity of SKOV3/PTX cells, however, KDM5A overexpression inhibited the PTX sensitivity of SKOV3 cells. In addition, CCK-8 and anchorage-independent growth assays demonstrated that KDM5A promoted cell proliferation. In addition, the FACS flow cytometer assay revealed that KDM5A promoted cell proliferation through facilitating cell cycle progression. However, the detailed molecular mechanisms of KDM5A have not been elucidated. KDM5A as a histone demethylase specific for H3K4 and it is required for cell development (12-14). The authors hypothesized that there may be several downstream target genes of KDM5A, and that these genes may regulate cell proliferation and the cell cycle.

Currently, cancer treatment options include chemotherapy, radiation therapy and surgical resection (25). For ovarian and lung cancer patients, PTX is one of the most commonly used anticancer drugs. However, there are multiple cancers are resistant to it (26). The authors explored the role of KDM5A in SKOV3/PTX cells. Although the target genes regulated by KDM5A that are involved in drug-resistance have not yet been identified, the expression of P-gp and the phenomenon of PTX sensitivity all conformed that KDM5A serve a key function in drug-resistance. Moreover, histone demethylases have been identified as an active frontier in epigenetic drug development (27,28). A previous study suggested that the drug-resistant mechanisms of carcinoma included anti-oncogene inactivation, oncogene activation, drug target molecular changes, reduced intracellular drug concentration, enhanced DNA damage repair function, metabolism detoxification and inhibition of tumor cells apoptosis. Drug-resistance is resulted by multiple genes, factors and a series of complex processes (29). The

detail mechanisms of KDM5A in PTX-resistance remain unknown, therefore, the authors have decided to next identify the downstream target gene of KDM5A, and further decipher the function of KDM5A in drug-resistance.

To the best of the authors' knowledge, the present study is the first to report that KDM5A mediates drug resistance in ovarian cancer, and increases ovarian carcinoma cell invasion ability and growth. The present work suggested that KDM5A was a critical regulator in ovarian cancer and may be a novel therapeutic target of ovarian cancer, especially in PTX-resistant cancer.

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