

Effects of cisplatin on the LSD1-mediated invasion and metastasis of prostate cancer cells

ZHI-YUAN CHEN, HUI CHEN, TAO QIU, XIAO-DONG WENG, JIA GUO, LEI WANG and XIU-HENG LIU

Department of Urology, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060, P.R. China

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Abstract. Prostate cancer poses a major public health problem in men. Metastatic prostate cancer is incurable, and ultimately threatens the life of patients. Lysine-specific demethylase 1 (LSD1) is an androgen receptor-interacting protein that exerts a key role in regulating gene expression and is involved in numerous biological processes associated with prostate cancer. Cisplatin, also known as *cis*-diamminedichloroplatinum or DDP, is a standard chemotherapeutic agent used to treat prostate cancer; however, it has the disadvantage of various serious side effects. The present study aimed to investigate the effects of LSD1 knockdown, and the interplay between LSD1 and DDP, on prostate cancer cell proliferation, apoptosis and invasion, and, therefore, the potential of LSD1 as a target for prostate cancer therapy. Flow cytometric analysis, Cell Counting kit 8 assay, Transwell assay and western blotting results revealed that LSD1 knockdown, in combination with DDP treatment, exerted antiproliferative, proapoptotic and anti-invasive effects on PC3 prostate cancer cells. In addition, knockdown of LSD1 acted synergistically with DDP, thereby enhancing the induction of apoptosis, and the inhibition of proliferation and invasion in prostate cancer cells. These results indicated that LSD1 may serve as a potential therapeutic target, and may enhance the sensitivity of PC3 cells to DDP.

Introduction

Prostate cancer, which is characterized by the development of prostate epithelial malignant tumors, occurs solely in men and is associated with the highest rates of morbidity and mortality (1). Prostate cancer poses a major public health problem worldwide (1). The incidence of prostate cancer is particularly prevalent in older men (2). The therapeutic

strategies currently used to treat prostate cancer include watchful waiting, surgery, radiotherapy, chemotherapy, hormone therapy and biotherapy (3). The androgen receptor (AR) has an important role in prostate cancer development and progression (3). Androgen-deprivation therapy (ADT) is an important means of treatment for patients with prostate cancer; however, one disadvantage is that the prostate cancer may develop resistance to ADT over time (4). At present, there is a dearth of effective treatment methods which are beneficial to those patients who have developed androgen resistance in prostate cancer. Therefore, prostate cancer therapy remains unsatisfactory, and there is an urgent requirement to identify novel therapeutic strategies to overcome resistance to androgens in patients with prostate cancer.

Lysine specific demethylase 1 (LSD1) is a histone demethylase, which exerts important roles in tumorigenesis (5-8). LSD1 has been reported to be highly expressed in various cancer cell types, particularly in prostate cancer (9). Previous studies have demonstrated that LSD1, as an AR-interacting protein, may promote AR-dependent gene expression, which subsequently leads to the constitutive maintenance of cancer cells via growth signals and an enhanced risk of tumor relapse (9,10). In addition, it has been suggested that histone modification patterns may be used to predict the risk of prostate cancer recurrence (11). Although LSD1 regulates the expression of a wide range of genes and is involved in the processes of prostate cancer progression and deterioration (9), the underlying molecular mechanisms remain to be fully elucidated. Therefore, the inhibition of LSD1 activity may provide a useful target for the treatment of prostate cancer.

Cisplatin, also known as *cis*-diamminedichloroplatinum or DDP, is a platinum-based drug commonly used in the clinic as a chemotherapeutic agent. It has numerous characteristic properties, including broad-spectrum anticancer activity and curative effects, which render it useful for the clinical treatment of various tumors (12). However, its use is associated with several side effects, which serve to limit the doses that may be administered, predominantly due to nephrotoxicity (13). Even so, it remains in use as a standard chemotherapeutic agent for the treatment of numerous types of cancer, including ovarian, cervical and prostate cancer (14-17). A previous study demonstrated that patients treated with DDP in combination with β -elemene were able to better tolerate the chemotherapy, which afforded an improved treatment for hormone-refractory prostate cancer (18).

Correspondence to: Mr. Zhi-Yuan Chen, Department of Urology, Renmin Hospital of Wuhan University, 238 Jiefang Road, Wuhan, Hubei 430060, P.R. China
E-mail: zhiyuanchen100@163.com

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Therefore, how to reduce the toxicity associated with DDP treatment is a keenly studied topic in cancer research.

The present study aimed to provide important insights into the effects of LSD1 knockdown and its interplay with DDP on the proliferation, apoptosis and invasion of PC3 human prostate cancer cells. In addition, the present study revealed whether LSD1 knockdown could increase the sensitivity of DDP for the treatment of prostate cancer. The results may provide important implications for the development of novel therapeutic strategies.

Materials and methods

Cell line and culture. The PC3 human prostate cancer cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in Gibco™ RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine (both Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified atmosphere containing 5% CO₂. Plasmids encoding LSD1 small interfering (si)RNA or mock vehicle pCMV-G&NR-U6-shRNA (GeneChem Co., Ltd., Shanghai, China) were transfected into the PC3 cells in 6-well plates using a lentiviral vector (JRDUN Biotechnology, Shanghai, China) and Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. Three sequences of the LSD1 were used, as follows: Short hairpin (sh)LSD1-1, 5'-ACGAAAGTG TCTCCGTTGA-3'; shLSD1-2, 5'-CCGACATGGCTTTCT CTTT-3'; and shLSD1-3, 5'-TCGACAGTGACCCCTTATA-3'. The cells were split twice weekly, and cells in the logarithmic growth phase were used for subsequent experiments.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and the mRNA was reverse transcribed into cDNA using the TIANScript RT kit (Tiangen Biotech. Co. Ltd., Shanghai, China). Subsequently, qPCR was conducted using the SYBR Green PCR Master mix (Thermo Fisher Scientific, Inc.) and the ABI 7300 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.), in which glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as the reference gene. The following primers were used: LSD1, forward (F) 5'-AAG CAGGAGGACTTCAAGAC-3', reverse (R) 5'-GCAGTG TGCGGTTTCTAATG-3'; GAPDH, F 5'-CACCCACTCCTC CACCTTTG-3' and R 5'-CCACCACCTGTTGCTGTAG-3' (Generay Biotech Co., Ltd., Shanghai, China). The PCR cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. RT-qPCR data were analyzed with SDS 2.3 software. Each experiment was repeated three times and the relative mRNA expression levels of LSD1 were calculated using the 2^{-ΔΔC_q} method (19).

Cell Counting kit 8 (CCK-8) assay. The viability of the PC3 cells was measured using a CCK-8 kit (Boster Biological Technology, Ltd., Wuhan, China). The cells were seeded into a 96-well microplate at a density of 5×10³/well and incubated for 24 h. The peripheral wells of the microplate contained phosphate-buffered saline (PBS) only. The cells were divided into four groups: Mock vehicle group, LSD1 siRNA group,

mock vehicle + DDP group or LSD1 siRNA + DDP group, according to the experimental design. The cells were treated with 5 μg/ml DDP (Beyotime Institute of Biotechnology, Haimen, China), after which the cells were incubated for a further 24 h prior to the assay. A total of 10 μl CCK8 solution was added to each well containing PC3 cells, and the cells were incubated for 0, 24, 48 or 72 h. Finally, the absorbance of each well was measured at 490 nm using a Gemini XPS microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Flow cytometric analysis. The PC3 cells (5×10⁵ cells/ml) were inoculated into 6-well plates. Each group comprised three double wells on the plate. Following a 24 h incubation, the groups were generated by addition of the appropriate reagents to the cells, and the cells were incubated for a further 24 h. The flow cytometric analysis was performed according to the protocol of the Annexin V-Fluorescein Isothiocyanate Apoptosis Detection kit (Abcam, Cambridge, UK). The apoptotic rates were analyzed immediately using a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA).

Cell invasive capability measured using a Transwell assay. Following the removal of the culture medium from each group, the cells were digested with trypsin and diluted to 1×10⁵/ml in serum-free Dulbecco's Modified Eagle's Medium containing 1% FBS (GE Healthcare Life Sciences, Logan, UT, USA). A total of 800 μl culture medium containing 10% FBS was added to the coated lower chambers of a Transwell system. Matrigel (BD Biosciences) was coated onto the upper chambers of the Transwell system, after which 200 μl cell suspension was added to the upper chambers at a density of 5×10⁴/well. The plate was cultured for 24 h, after which the cells on the upper layer were removed. The cells that had migrated to the lower layer were washed with PBS, fixed with methanol and stained with 1% crystal violet. The invasive cells were counted in five fields for each sample under an inverted microscope (BX51; Olympus Corporation, Tokyo, Japan), and the results were averaged. The experiments were repeated three times.

Western blot analysis. Total protein was extracted from the cell samples using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology), and was quantified using the Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific, Inc.). Subsequently, equal volumes of protein (30 μg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting onto a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA) using an electrophoretic transfer cell (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked with 5% skimmed milk, followed by incubation overnight at 4°C with the following primary antibodies: Monoclonal anti-E-cadherin (#14472; 1:1,000), polyclonal anti-cyclo-oxygenase-2 (COX-2; #4842; 1:1,000), monoclonal anti-Smad2/3 (#8685; 1:1,000), monoclonal anti-phosphorylated (p)-Smad2/3 (#8828; 1:1,000), monoclonal anti-cyclin D (#2978S; 1:1,000), polyclonal anti-p-Akt (#9271; 1:1,000), polyclonal anti-Akt (#9272; 1:1,000), monoclonal anti-extracellular signal-regulated kinase (ERK; #4695; 1:1,000), monoclonal anti-p-ERK (#4376;

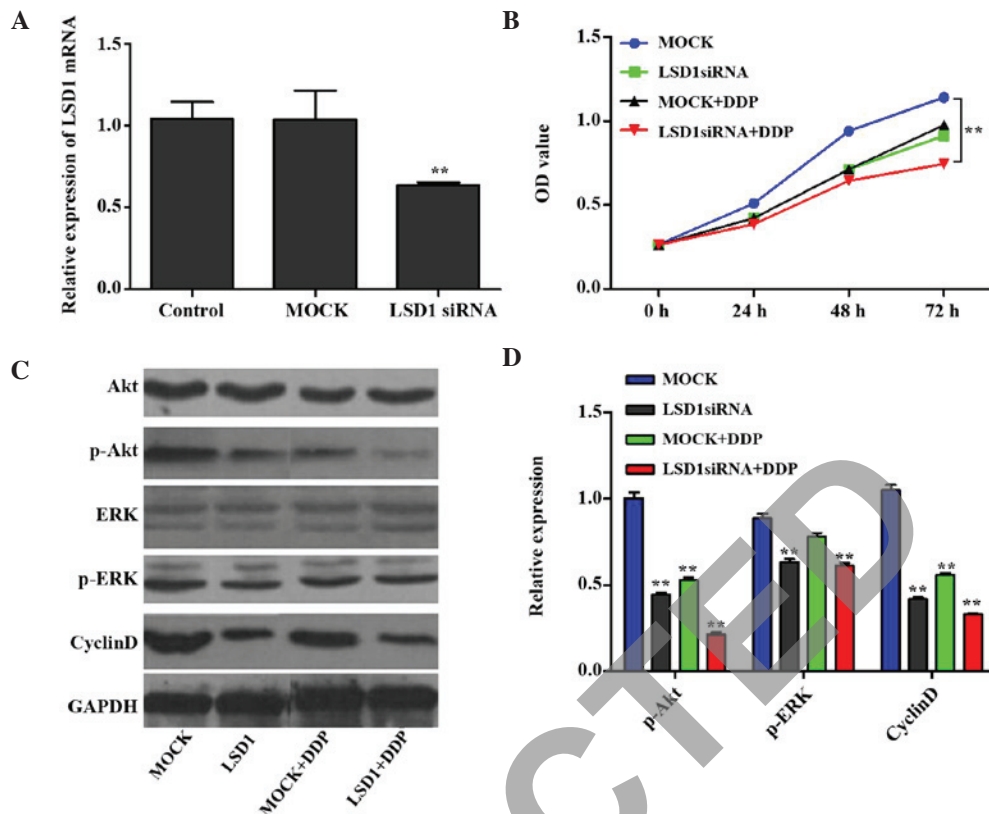


Figure 1. LSD1 RNA interference and cell growth rate. (A) Compared with the control group, significantly decreased mRNA expression levels of LSD1 were detected following LSD1 knockdown; however, no significant changes were observed in the MOCK group (control cells transfected with an irrelevant interference sequence). (B) Cell growth in each group was measured using the Cell Counting kit-8 assay. At 0 h, all groups exhibited very similar cell numbers, whereas at 24, 48 and 72 h, the growth rate of the PC3 cells was significantly decreased following LSD1 knockdown, as compared with the MOCK group. Similar results were observed in the DDP group. Notably, the combined action of LSD1 knockdown and DDP inhibited PC3 cell proliferation more markedly, as compared with either considered in isolation. (C and D) Protein expression levels of Akt, ERK and cyclin D are shown. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. the control or MOCK group. LSD1, lysine specific demethylase 1; siRNA, small interfering RNA; DDP, *cis*-diamminedichloroplatinum/cisplatin; OD, optical density; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ERK, extracellular signal-regulated kinase.

1:1,000) and monoclonal anti-GAPDH (#5174; 1:1,500) from Cell Signaling Technology, Inc. (Danvers, MA, USA), and polyclonal anti-transforming growth factor- β 1 (TGF- β 1; ab92486; 1:800) and anti-vascular endothelial growth factor (VEGF; ab46154; 1:1,000) from Abcam. Subsequently, the blots were washed three times with PBS and incubated with horseradish peroxidase-conjugated goat anti-mouse (A0216; 1:1,000) or goat anti-rabbit (A0208; 1:1,000; both Beyotime Institute of Biotechnology) secondary antibodies for 1 h at room temperature. The bands were detected by reaction with enhanced chemiluminescence detection system reagents (EMD Millipore) and exposure to X-ray film (Kodak, Rochester, NY, USA), which was subsequently developed and used to capture photographic images. GAPDH was used to normalize the protein expression. Band intensities were analyzed using ImageJ 1.49 software (<https://imagej.nih.gov/ij/>).

Statistical analysis. All data are presented as the mean \pm standard deviation. The data were evaluated using the Prism 5.0 statistical software package (GraphPad Software Inc., San Diego, CA, USA). The two-tailed Student's *t*-test was used to evaluate statistical differences between two groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Proliferation of PC3 cells is decreased following LSD1 knock-down and treatment with DDP. The present study hypothesized that LSD1 may have an important role in PC3 cells, which has not been previously investigated. Lentiviral-mediated RNA interference technology was used to establish a stably transfected LSD1 knockdown PC3 cell line, and the CCK-8 colorimetric assay was subsequently used to determine cell proliferation. As shown in Fig. 1A, the mRNA expression levels of LSD1 in the knockdown group were decreased to $<60\%$ of the control levels ($P < 0.01$), whereas the mRNA expression levels of LSD1 in the mock group (i.e. control cells which were transfected with an irrelevant interference sequence) exhibited no significant changes. All of the groups contained similar cell numbers at the 0 h time point; however, proliferation of the LSD1 knockdown PC3 cells was significantly decreased after 24, 48 and 72 h, as compared with the mock group ($P < 0.01$; Fig. 1B). Furthermore, compared with the mock group, DDP (at a concentration of $5 \mu\text{g/ml}$) exerted a marked inhibitory effect on PC3 cell proliferation. Notably, the LSD1 knock-down + DDP group demonstrated a more marked inhibition on PC3 cell proliferation, as compared with the DDP or LSD1 knockdown groups ($P < 0.05$). These findings indicate that the proliferative capability of the PC3 cells was decreased

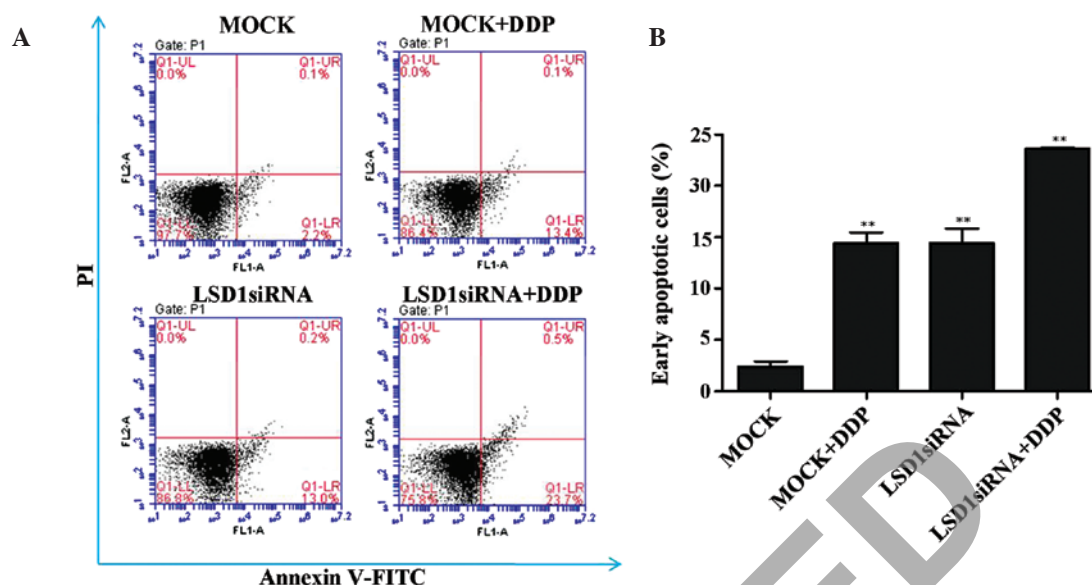


Figure 2. Changes in the percentage of PC3 apoptotic cells following RNA interference. Cell apoptosis in each group was detected using Annexin V/PI staining. (A) Flow cytometric analysis of apoptosis. (B) Statistical analysis of the data obtained from the flow cytometric experiments. The proportion of PC3 cells in early apoptosis was increased following LSD1 siRNA-mediated knockdown or DDP treatment, as compared with the MOCK group. Data are presented as the mean \pm standard deviation ($n=3$). ** $P<0.01$, compared with the MOCK group. LSD1, lysine specific demethylase 1; siRNA, small interfering RNA; DDP, cis-diamminedichloroplatinum/cisplatin; FITC, fluorescein isothiocyanate; PI, propidium iodide.

following LSD1 knockdown, thus suggesting that LSD1 may contribute to the proliferation of PC3 cells, and that siRNA interference may result in reduced cell growth. Furthermore, DDP had similar effects to LSD1 siRNA, and a combination of LSD1 knockdown and DDP treatment produced a synergistic effect.

LSD1 knockdown and DDP treatment inhibit PC3 cell proliferation via regulation of the Akt and ERK signal transduction pathways. The results of the present study revealed that cell proliferation was inhibited when LSD1 was knocked down and the cells were co-treated with DDP, as compared with the mock group. Since the Akt and ERK signal transduction pathways are reported to modulate cell proliferation and tumorigenicity (20,21), the protein expression levels of p-Akt and p-ERK were subsequently investigated. Furthermore, cyclin D has a crucial role in the Akt signal pathway as a regulatory protein of the cell cycle (22), and therefore the protein expression levels of cyclin D were also investigated. The protein expression levels of p-Akt, p-ERK and cyclin D1 were downregulated in the treatment groups (LSD1, mock + DDP, LSD1 + DDP), as compared with the mock group (Fig. 1C and D). These results suggest that LSD1 RNA interference and treatment with DDP may inhibit PC3 cell proliferation via regulation of the Akt and ERK signal pathways.

Percentage of PC3 cells in the early apoptotic phase is increased following LSD1 knockdown and treatment with DDP. In addition to regulating PC3 cell growth, LSD1 may be involved in regulating cell apoptosis. As shown in Fig. 2A and B the proportion of PC3 cells in the early apoptotic phase was significantly increased following LSD1 knockdown, as compared with the mock group. Furthermore, it was revealed that DDP

contributes to the increased levels of apoptosis in the PC3 cells, and that treatment with DDP exerted a synergistic action on PC3 cell apoptosis when combined with LSD1 siRNA. These results suggest that a combination of LSD1 knockdown and DDP treatment may contribute to the increased percentage of PC3 cells in the early apoptotic stage.

Invasive capability of PC3 cells is decreased following LSD1 knockdown and treatment with DDP via regulation of the TGF- β 1/Smad2/3 signal transduction pathway. Invasive capability is essential for the malignant progression of tumors. As shown in Fig. 3A and B, the invasive capability of the PC3 cells was decreased following LSD1 knockdown or treatment with DDP, as compared with the mock group. Furthermore, a combination of LSD1 knockdown and DDP treatment exerted a synergistic effect on the decline in invasive ability. These results suggest that LSD1 may have a crucial role in the invasion of PC3 cells.

TGF- β 1 is a cytokine peptide, which is associated with various biological roles in cancer (23). The Smad proteins act as substrates of the TGF- β 1 receptor, and their activation by phosphorylation propagates the TGF- β 1 signal transduction. In particular, the TGF- β 1/Smad2/3 signaling pathway is involved in mechanisms underlying the invasion and metastasis of prostate cancer cells, a process which is also regulated by tumor angiogenesis, the host immune system, the cells themselves and the surrounding matrix microenvironment (24-26). Consequently, the protein expression levels of TGF- β 1, p-Smad2/3, VEGF, COX-2 and E-cadherin, all of which are associated with the TGF- β 1/Smad2/3 signal transduction pathway, were investigated. The results revealed that the expression levels of TGF- β 1, p-Smad2/3, VEGF and COX-2 in the DDP-treated cells were downregulated, whereas the protein expression levels of E-cadherin were upregulated.

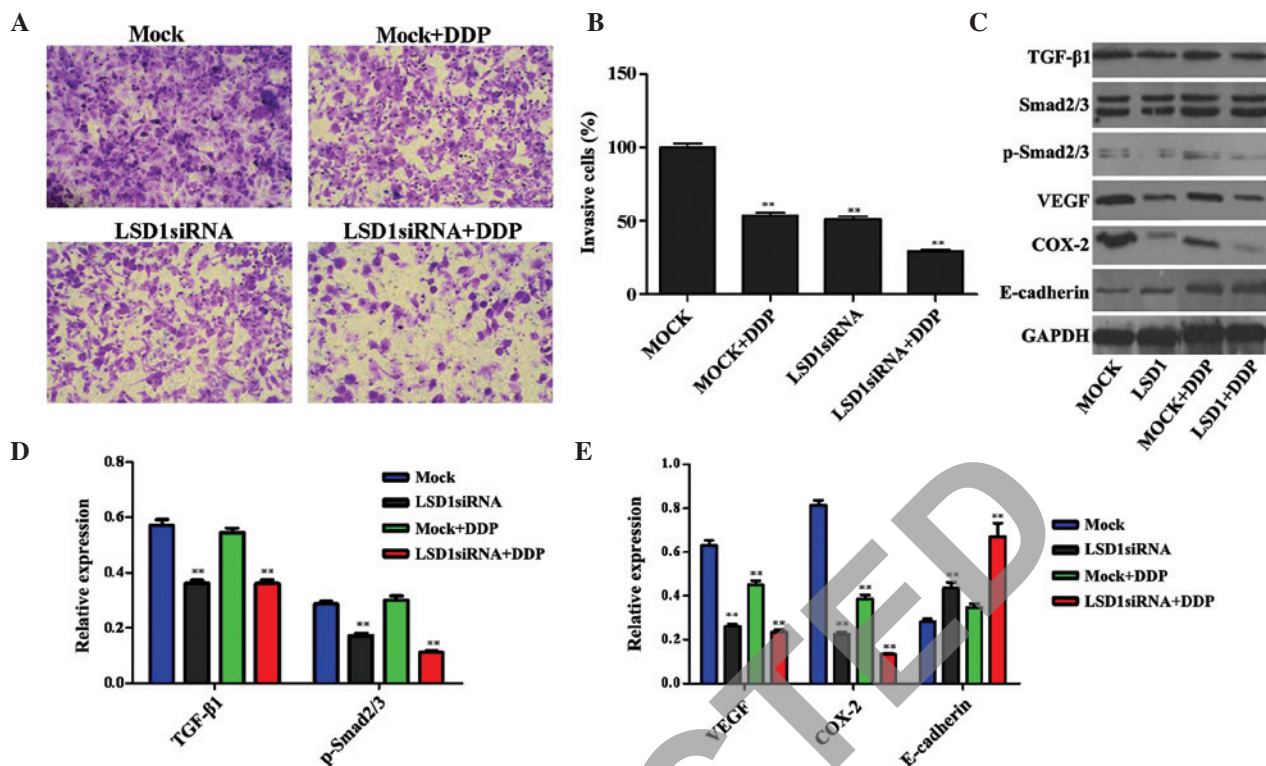


Figure 3. Changes in the invasive capability of PC3 cells following LSD1 RNA interference were detected using the Transwell method. (A and B) Images are shown detailing cellular invasion of the various cell groups (magnification, x200). Compared with the MOCK group, the invasive capability of the PC3 cells was decreased following LSD1 knockdown or DDP treatment. Furthermore, LSD1 knockdown and treatment with DDP exerted a synergistic effect on the decrease in invasive capability. (C) Expression levels of proteins associated with the TGF- β 1/Smad2/3 signaling pathway were detected. (D and E) Quantification of the western blotting data. Data are presented as the mean \pm standard deviation. ** $P < 0.01$, compared with the MOCK group. LSD1, lysine specific demethylase 1; siRNA, small interfering RNA; DDP, cis-diamminedichloroplatinum/cisplatin; VEGF, vascular endothelial growth factor; COX-2, cyclo-oxygenase-2; TGF- β 1, transforming growth factor- β 1; p-, phosphorylated; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Furthermore, the combination of LSD1 knockdown and treatment with DDP exerted a synergistic effect, as compared with either treatment taken in isolation or with the mock cells (Fig. 3C-E).

Discussion

Prostate cancer is a complex disease, and numerous controversies are associated with aspects of different treatment strategies (27). The identification of genetic and molecular events that may improve the early detection of prostate cancer, or that could be used as therapeutic targets, is a top priority in this line of study.

LSD1 is a flavin-dependent amine oxidase, which has been reported to interact with the AR (9). Previous studies have reported that LSD1 is overexpressed in prostate cancer (9,28). DDP is an inorganic compound that is widely used in cancer therapy (12). In the present study, the antiproliferative, proapoptotic and anti-invasive effects of LSD1 knockdown and DDP treatment, either in isolation or in combination, on PC3 prostate cancer cells were investigated. The results of the present study may lead to an improved understanding regarding how LSD1 knockdown and DDP treatment affect the physiological activity of prostate cancer cells, thereby providing a novel target for therapeutic intervention in prostate cancer.

A combination of LSD1 knockdown and DDP treatment effectively suppressed the proliferation of PC3 cells. The Akt

and ERK signaling pathways have been reported to be important pathways closely associated with cell proliferation (29,30). In the present study, western blotting results suggested that the protein expression levels of the associated proteins, p-Akt, cyclin D1 and p-ERK, were markedly decreased in the LSD1 siRNA, DDP treatment and LSD1 siRNA + DDP cell groups, as compared with the mock group. These findings indicated that LSD1 knockdown and DDP treatment may effectively inhibit the proliferation of PC3 cells via regulation of the Akt and ERK signaling pathways. Flow cytometry was also performed to determine the extent of apoptosis in the prostate cancer cells. All of the treatments, i.e. LSD1 knockdown and treatment with DDP, either alone or in combination, resulted in an increased induction of PC3 cell apoptosis.

Tumor cell invasion and metastasis are complicated by the existence, and interplay, of several mechanisms (31). The effects of LSD1 knockdown on the invasive capability of the PC3 prostate cancer cells were examined in the present study. The results demonstrated that knockdown of LSD1, in combination with DDP, suppressed the invasion of prostate cancer cells. TGF- β is a cytokine peptide, which exerts various biological activities. A previous study demonstrated that TGF- β 1 was able to promote the invasion and metastasis of prostate tumor cells (32). Smad proteins are the sole substrates of TGF- β 1 identified in the TGF- β 1 signal transduction pathway (33). In the present study, the protein expression levels of TGF- β 1 and p-Smad2/3 were decreased, which implied that this signaling

pathway may be primarily responsible for the process by which LSD1 knockdown and DDP treatment inhibited the invasion of PC3 cells. TGF- β 1/Smad2/3 signaling is known to promote specific mechanisms underlying the processes of cell invasion and metastasis in prostate cancer, via regulation of angiogenesis, the immune defense system, changes in the substrate microenvironment or alterations to the cells themselves (34-36).

The formation of blood vessels is crucial in tumor growth, invasion and metastasis (37). The present study revealed that VEGF, as one of the most important promoters of angiogenesis (38), was downregulated in the LSD1 siRNA group. In addition, epithelial-mesenchymal transition is considered to be a key step in the process of tumor metastasis. For example, E-cadherin is as a Ca²⁺-dependent glycoprotein, which performs an essential role in cell invasion and migration (39,40). The results of the present study implied that LSD1 knockdown and DDP treatment inhibited the invasion of the PC3 cells by upregulating the expression levels of E-cadherin. COX-2, which is a rate-limiting enzyme in the prostaglandin biosynthesis pathway, is able to promote tumor angiogenesis (41). The western blotting results of the present study demonstrated that the protein expression levels of COX-2 were decreased in DDP-treated LSD1 knockdown cells, with a similar result obtained for VEGF. These results may verify the hypothesis that LSD1 knockdown inhibits the invasion of PC3 cells by regulating the TGF- β 1/Smad2/3 signal pathway, and a combination of LSD1 siRNA and DDP treatment may lead to more pronounced effects.

In conclusion, the present study aimed to identify the proapoptotic, antiproliferative and anti-invasive effects of LSD1 knockdown, in combination with DDP treatment, on PC3 human prostate cancer cells, and to offer an explanation for the underlying mechanism. The results of the present study may provide novel insights into the molecular mechanism underlying the progression and pathogenesis of prostate cancer, and may be useful for the optimization of therapeutic interventions for the treatment of this disease.

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RETRACTED