LSD1-mediated epigenetic modification contributes to ovarian cancer cell migration and invasion

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Abstract. Lysine-specific demethylase 1 (LSD1) has been implicated in the process of tumor progression at various steps, but its role in epithelial-messenchymal transition (EMT) and the migration of ovarian cancer cells remains obscure. In this study, we demonstrated the effect of LSD1 on ovarian cancer cell migration and the regulatory role of LSD1 in the expression of EMT markers. Inhibition of LSD1 expression impaired the migration and invasion of HO8910 ovarian cancer cells. In contrast, overexpression of LSD1 enhanced the cell migration and invasion of HO8910 cells. Mechanistic analyses showed that LSD1 promoted cell migration through induction of N-cadherin, vimentin, MMP-2 and inhibition of E-cadherin. Furthermore, LSD1 interacted with the promoter of E-cadherin and demethylated histone H3 lysine 4 (H3K4) at this region, downregulated E-cadherin expression, and consequently enhanced ovarian cancer cell migration. These data indicate that LSD1 acts as an epigenetic regulator of EMT and contributes to the metastasis of ovarian cancer.

Introduction

Ovarian cancer is the second most common cancer among female gynecologic cancers and has become the leading cause of cancer-related death among females (1). Due to the difficulty in early detection, 75% of ovarian cancer patients are diagnosed at advanced stages (stage III or IV) (2). In stage III or IV, the tumor involves one or both ovaries with peritoneal metastasis outside the pelvis or distant metastasis to liver parenchyma or other visceral organs (2,3). Early invasion and metastasis have been well accepted as the leading features and main causes of death in ovarian cancer. However, mechanistic understanding of the metastatic potential of ovarian cancer remains unclear, and novel targets are yet to be identified for treating metastatic ovarian cancer.

Lysine-specific demethylase 1 (LSD1/KDM1A/AOF2) is the first histone demethylase discovered, which specifically demethylates mono- and dimethylated histone H3 lysine 4 (H3K4) and histone H3 lysine 9 (H3K9) (4). LSD1 is frequently overexpressed in lung cancer (5,6), breast cancer (7), prostate cancer (8,9), and liver cancer (10). Importantly, overexpression of LSD1 promotes the growth and invasion of various types of cancer cells, and contributes to human carcinogenesis by regulating the expression of genes involved in various chromatin-modifying pathways (6). Conversely, inhibition of LSD1 was found to suppress cell invasion and migration in various types of cancers (5,11,12). Although LSD1 is recently described to be highly expressed in ovarian cancer (13,14), the biological function of LSD1 in this cancer remains largely unknown.

Epithelial-messenchymal transition (EMT) is a process whereby epithelial cells are programmed into mesenchymal cells (15). EMT is now considered as the initial and essential step in tumor metastasis. During EMT, epithelial cells acquire cell motility by reducing cell-cell junctions, and loss of cell polarity (16,17). E-cadherin, an epithelial marker, has a crucial role in regulating cell-cell adhesion and maintenance of tissue architecture (18). Indeed, E-cadherin serves as a suppressor of cell migration and invasion (19-22). Transcription factors, including Snail, Slug, Zeb1 and Twist, can induce EMT by downregulating E-cadherin expression (23-26). Recent studies show that LSD1 is recruited by the transcription factor Snail to the promoter of E-cadherin to repress the expression of the E-cadherin gene consequently contributing to cancer cell invasion (27,28). Conversely, Ferrari-Amorotti *et al* observed that blocking Snail-LSD1 interaction by treatment with Parnate suppressed the invasiveness of cancer cells (29).

Few studies have reported on how LSD1 induces EMT and finally contributes to ovarian cancer cell migration. Therefore in the present study, we examined the effect of LSD1 on cell migration and invasion using LSD1-knockdown and

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overexpressing HO8910 ovarian cancer cells as models. We also examined the regulatory role of LSD1 in the expression of molecular markers of EMT. Knockdown of LSD1 reduced cell migration and invasion in the HO8910 cells, while overexpression of LSD1 stimulated the migration and invasion of the HO8910 cells. Mechanistic analyses uncovered that LSD1 promoted cell migration through induction of N-cadherin, Snail, vimentin, MMP-2 and inhibition of E-cadherin. LSD1 epigenetically regulated the transcription of E-cadherin through demethylating H3K4 at the E-cadherin promoter. Collectively, these results suggest that targeting LSD1 may be a novel therapeutic approach for the treatment of ovarian cancer.

Materials and methods

Cell lines and cell culture. The human ovarian cancer cell line, HO8910, was kindly provided by Dr Qixiang Shao of Jiangsu University (Zhenjiang, China). HO8910 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (both from Gibco, Grand Island, NY, USA) at a temperature of 37˚C under 5% CO_2. HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% FBS at a temperature of 37˚C under 5% CO_2.

Antibodies and reagents. The pLKO-Tet-On, pLVX-tight-puro, pHR'-CMV-8.2ΔVPR, and pHR'-CMV-VSVG vectors were kind gifts from Dr Changdeng Hu (Purdue University, West Lafayette, IN, USA). LSD1, E-cadherin, Snail, vimentin, N-cadherin and MMP-2 antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). The α-tubulin and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies were obtained from Bioworld Technology (Shanghai, China). Electrochemiluminescence (ECL) reagents were purchased from Millipore Corp. (Billerica, MA, USA). H3K4me2 antibody was purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Polybrene, doxycycline (Dox), puromycin and G418 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The LSD1 inhibitor tranylcypromine (TCP) was obtained from Biomol International (Plymouth Meeting, PA, USA).

Plasmid constructions and transfections. For generation of the shRNA-LSD1 plasmid, annealed short hairpin oligonucleotides (the RNAi Consortium collection TRCN0000046072; Sigma-Aldrich) targeting CCACGAGTCAAACCTTTATTT in the coding regions (CDS) of LSD1 were cloned into pLKO-Tet-On by *Age*I and *Eco*RI sites to produce pLKO-Tet-On-shLSD1 as described previously (30,31). The constructs were confirmed by DNA sequencing. All transfections were performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Establishment of the stable cell lines (LSD1-knockdown and overexpressing). To generate lentiviral particles, 293T cells were seeded in 6-cm dishes and transfected with 2 μg of pLKO-Tet-On-shLSD1, 1.5 μg of pHR'-CMV-8.2ΔVPR and 0.5 μg of pHR'-CMV-VSVG using Lipofectamine 2000 reagent. The supernatant containing the lentiviral particles was harvested 24, 48 and 72 h post-transfection, and then centrifuged (124 x g for 5 min) to remove cell debris. HO8910 cells cultured in 6-cm dishes were infected by adding 1 ml lentiviral supernatant and 3 ml complete medium containing 8 μg/ml Polybrene. After the infection (twice), cells were selected with 2.0 μg/ml puromycin for 3 days and then maintained with 1.0 μg/ml puromycin for one week.

To generate rTet-repressor expressing (rtTA) cell line, 293T cells were transfected with 2 μg of pLVX-Tet-On, 1.5 μg of pHR'-CMV-8.2ΔVPR and 0.5 μg of pHR'-CMV-VSVG using Lipofectamine 2000 reagent. After transfection (24 h), the viral supernatant was harvested and used to infect HO8910 cells. After the infection (twice), HO8910 cells were selected with 200 μg/ml G418 for 1 week. The cells that survived were stable rtTA. HO8910-rtTA cells were then infected with the lentiviral particles packaged with pLVX-tight-puro-LSD1. After infection twice, HO8910-rtTA cells were selected with 2.0 μg/ml of puromycin for 3 days, and then maintained in the presence of 1.0 μg/ml of puromycin for one week. The surviving cells were considered as stable clones. The stable clones were further confirmed by western blot analysis.

RNA extraction and real-time RT-PCR (qRT-PCR). Total RNA was isolated from the cells using RNAiso Plus (Takara, Shiga, Japan) and reverse-transcribed using the PrimeScript RT reagent kit (Takara) to generate cDNAs. Then the cDNAs were subjected to qRT-PCR as described previously (32). qRT-PCR was performed with SYBR-Green PCR Master Mix (Takara) on a Bio-Rad CFX96 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primer sequences used were: LSD1 (GenBank accession no. NM 015013.3), 5'-CAAGTGTCAATTTGTTCGGG-3' (forward) and 5'-TTCTTTGGGCTGAGGTACTG-3' (reverse); and GAPDH (GenBank accession no. NM001256799.1), 5'-GCAAATTCCATGGCACCGTC-3' (forward) and 5'-TCGCCCCACTTGATTTTGG-3' (reverse). The relative quantification of mRNA levels was normalized to levels of GADPH and calculated by comparative $2^{-\Delta\Delta Ct}$.

Western blot analysis. Protein lysates were extracted from the cells and blotted as described previously (33). Equal amounts of soluble proteins were electrophoresed by SDS-PAGE and transferred to 0.45-μm PVDF membranes. The membranes were blocked with 5% nonfat-dry milk for 1 h at room temperature (RT). After incubation with the primary antibodies against LSD1 (1:1,000), E-cadherin (1:500), Snail (1:500), vimentin (1:500), N-cadherin (1:500) or MMP-2 (1:500) overnight at 4˚C and with the corresponding secondary antibodies (1:5,000) for 1 h at RT, the immunoblots were developed by ECL method.

Migration and invasion assays. For the invasion assay, each Boyden chamber (BD Biosciences, Bedford, MA, USA) was coated with 60 μl Matrigel diluted with DMEM (1:30) and incubated at 37˚C for 4-6 h. Cells ($1.5x10^5$) were resuspended with DMEM containing Dox or TCP in the upper chamber. Then, 10% FBS-containing medium was placed in the lower chamber to act as a chemoattractant. After a 24-h incubation, the non-invading cells remaining on the upper surface were removed, and the cells on the lower surface were fixed with 4% formaldehyde for 30 min, and stained with 0.1% crystal violet for 15 min. At least 5 fields for each chamber were

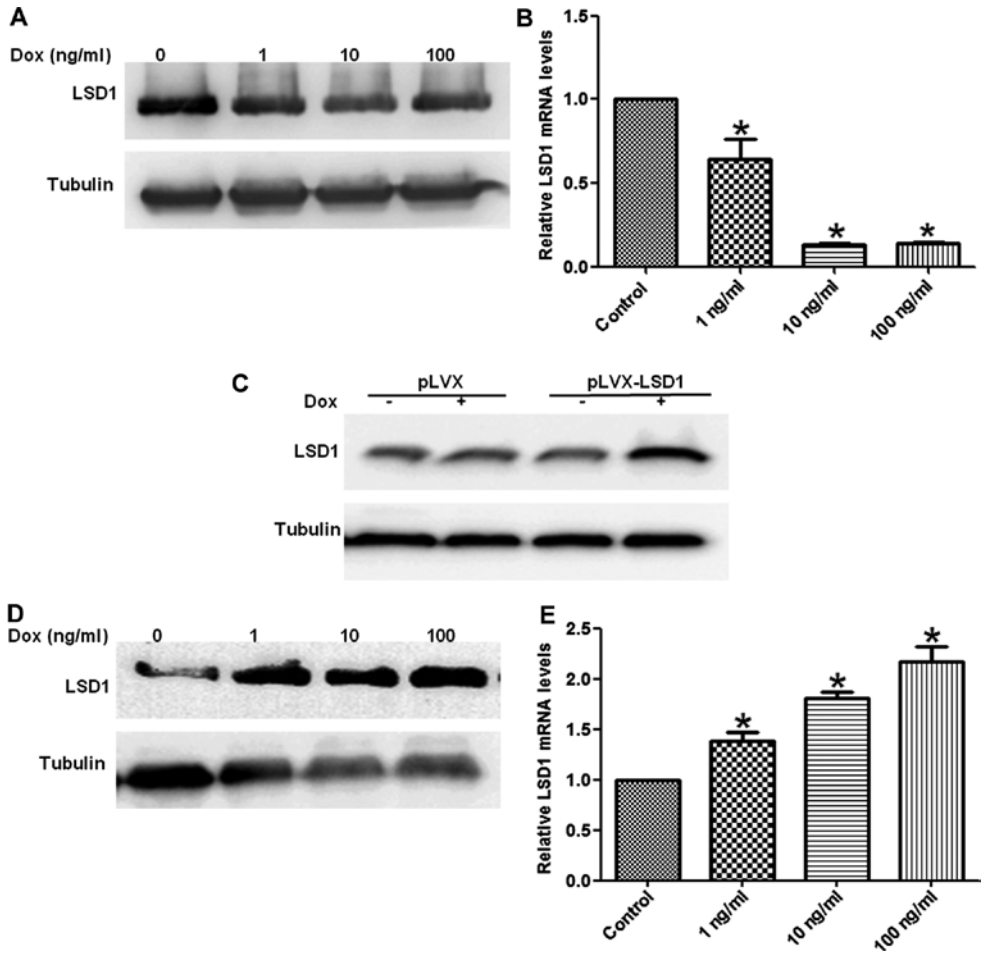

Figure 1. Verification of stable LSD1-KD and LSD1-OE HO8910 ovarian cancer cells following treatment with doxyxycline (Dox). (A and B) LSD1-KD HO8910 cells were treated with different doses of Dox for 48 h, after which LSD1 protein and mRNA levels were detected via western blotting (A) and qRT-PCR (B). (C) HO8910-pLVX empty vector and pLVX-LSD1 (LSD1-OE) cells were treated with or without 100 ng/ml Dox for 48 h, followed by immunoblotting analysis of LSD1 expression. (D and E) LSD1-OE HO8910 cells were treated with different doses of Dox for 48 h, after which LSD1 protein and mRNA levels were detected by western blotting (D) and qRT-PCR (E). α-tubulin was used as a loading control. The transcript levels of the LSD1 gene were normalized against those of GADPH and the value for the untreated control was set as 1. The bars of the histograms represent the mean ± SEM (n=4). *P<0.01.

photographed (x200 magnification) and counted, and the invading cells were counted in each field. The cell migration assay was performed using Boyden chambers without Matrigel coating. All experiments were performed at least in triplicate.

Chromatin immunoprecipitation (ChIP). All reagents were provided by Upstate Biotechnology (EZ-ChIP™ kit 17-371). Cells were fixed with 1% formaldehyde to cross-link proteins. The reaction was stopped by adding 10X glycine. Cross-linked cells were washed with PBS twice, pelleted and resuspended in SDS lysis buffer at a concentration of $1x10^7$ cells/ml. Aliquots of 400 μl were sonicated with 4-6 sets of 5-sec pulses (32% output) on ice. Then sonicated lysates were centrifuged and divided into 100 μl aliquots for each ChIP assay ($1x10^6$ cells/IP), and precleared with protein G-agarose. After incubation with the antibodies overnight at 4˚C, immune complexes were collected with protein G-agarose, and then washed with low salt immune complex wash buffer, high salt immune complex wash buffer, and finally TE buffer. The immune complexes were eluted with 20% SDS, and 1 M $NaHCO_3$. The crosslinks were reversed overnight at 65˚C, then the DNA was purified using spin columns, and finally subjected to qRT-PCR. Chromatin eluted from the IPs with IgG and anti-RNA polymerase were used as the negative and positive control, respectively. Two previously described primers of E-cadherin promoter for ChIP (34,35) were as follows: E-ca01 5'-GGGCAATACAGGGAGACACA-3' (forward) and 5'-GGGCTTTTACACTTGGCTGA-3' (reverse); E-ca02 5'-CACAACAGCATAGGGAGACATT-3' (forward) and 5'-TGTAGAGCTTCATGGGTTAGTGA-3' (reverse).

Statistical analysis. All values are presented as the mean ± SEM. The data were analyzed using the Student's t-test with SPSS 11.5 software (SPSS Inc.). P-values with a 95% confidence interval were obtained from at least three independent experiments. A P-value <0.01 was considered to indicate a statistically significant result.

Results

LSD1 is required for cell migration and invasion in ovarian cancer cells. To investigate the contribution of LSD1 to the

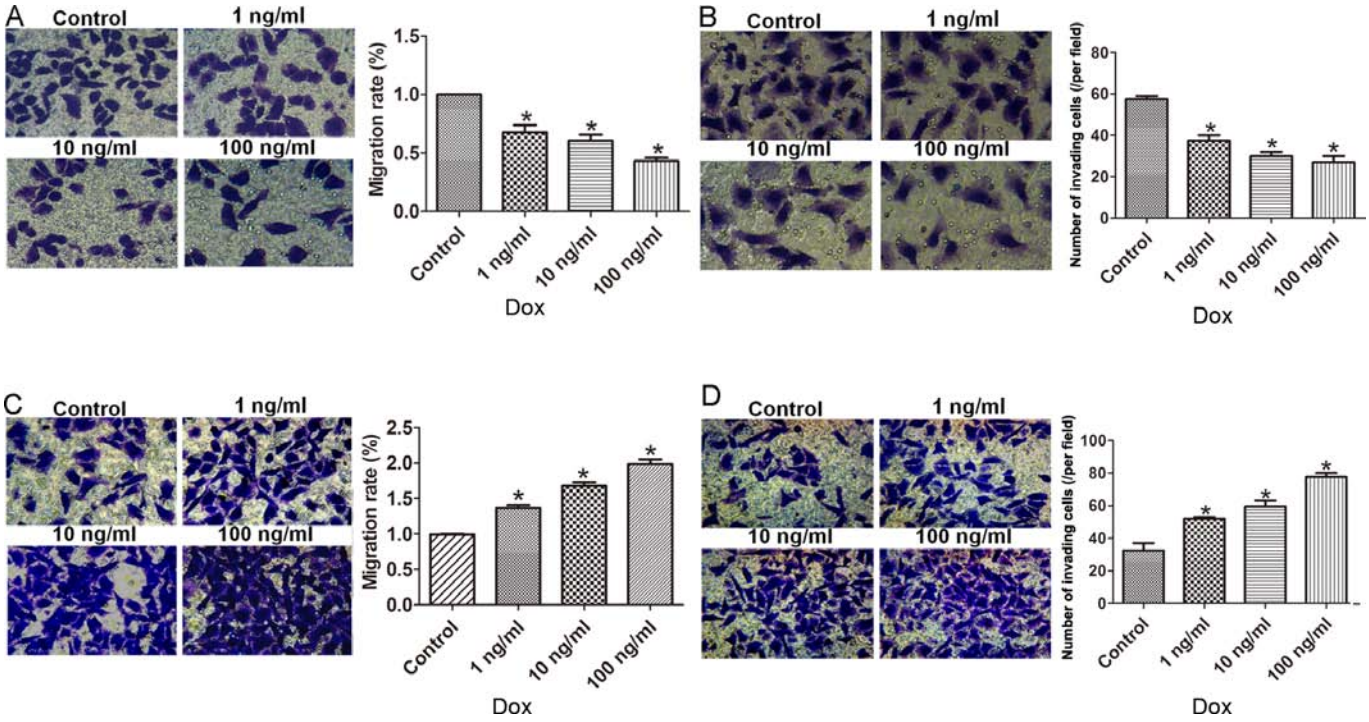

Figure 2. LSD1 is required for HO8910 cell migration and invasion. LSD1-KD HO8910 and LSD1-OE HO8910 cells were seeded in Boyden chambers without or with Matrigel, and cultured with different doses of doxorubicin hydrochloride (Dox) for 18 h. (A) The left panel shows representative images of the migrated LSD1-KD HO8910 cells (x200 magnification), and the right panel shows the migration rate. (B) The left panel shows representative images of the invading LSD1-KD HO8910 cells (x200 magnification), and the right panel shows the quantification of the average number of invasive cells per well. (C) The left panel shows representative images of the migrated LSD1-OE HO8910 cells (x200 magnification), and the right panel shows the migration rate. (D) The left panel shows representative images of the invading LSD1-OE HO8910 cells (x200 magnification), and the right panel shows the quantification of the average number of invasive cells per well. These experiments were repeated at least three times. The bars of the histograms represent the mean ± SEM (n=4). *P<0.01.

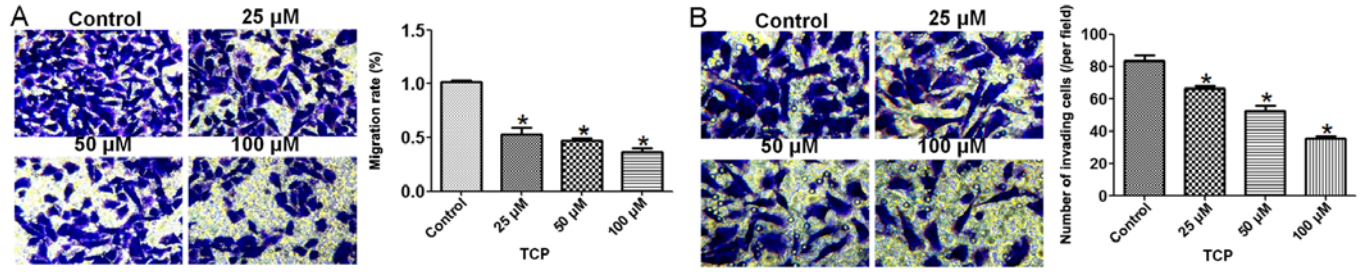

Figure 3. Tranylcypromine suppresses HO8910 cell migration and invasion. HO8910 cells were seeded in Boyden chambers without or with Matrigel, and cultured with different doses of tranylcypromine (TCP) for 24 h. (A) The left panel shows representative images of the migrated cells (x200 magnification), and the right panel shows the migration rate. (B) The left panel shows representative images of invasive cells (x200 magnification), and the right panel shows the quantification of the average number of invasive cells per well. The bars of the histograms represent the mean ± SEM (n=4). *P<0.01.

migration and invasion of ovarian cancer HO8910 cells, we generated stable LSD1-knockdown (LSD1-KD) clones and LSD1-overexpressing (LSD1-OE) clones from the HO8910 cells. Total RNA and proteins were extracted from these stable cells treated with increasing doses of Dox for 24 or 48 h. Our results showed the mRNA and protein expression of the LSD1 gene was decreased in the LSD1-KD cells in a dose-dependent manner (Fig. 1A and B), whereas the levels of LSD1 mRNA and protein expression were increased in the LSD1-OE cells (Fig. 1C-E).

To understand the effect of LSD1 expression on cell migration and invasion, we performed Transwell assays to measure the migratory capacity of these two transfected cell lines. The LSD1-KD cells displayed less migration and invasion in comparison with the control (Fig. 2A and B), whereas the LSD1-OE cells had a higher rate of migration and invasion as compared to the control (Fig. 2C and D).

To further determine the role of LSD1 in cell migration, we utilized a known potent inhibitor, TCP (30,36), to suppress the demethylase activity of LSD1 in HO8910 cells. Inhibition of LSD1 decreased the migration activity of the HO8910 cells in a dose-dependent manner (Fig. 3A and B). Taken together, these data suggest that LSD1 is essential for cell migration and invasion in HO8910 ovarian cancer cells.

LSD1 regulates EMT in ovarian cancer cells. As epithelial-mesenchymal transition (EMT) is involved in tumor migration and invasion, we examined the expression of several

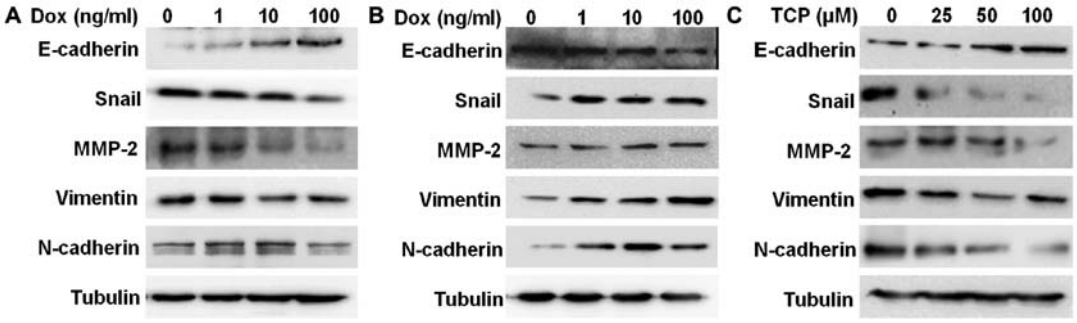

Figure 4. LSD1 regulates epithelial-messenchymal transition (EMT) in HO8910 cells. (A) LSD1-KD HO8910 and (B) LSD1-OE HO8910 cells were cultured with different doses of doxorubicin hydrochloride (Dox) for 48 h, after which the expression of EMT markers was analyzed by western blot analysis. (C) HO8910 cells were treated with different doses of tranylcypromine (TCP) for 48 h, and then the expression of EMT markers was detected via western blot analysis. α-tubulin was used as a loading control. This experiment was repeated at least three times.

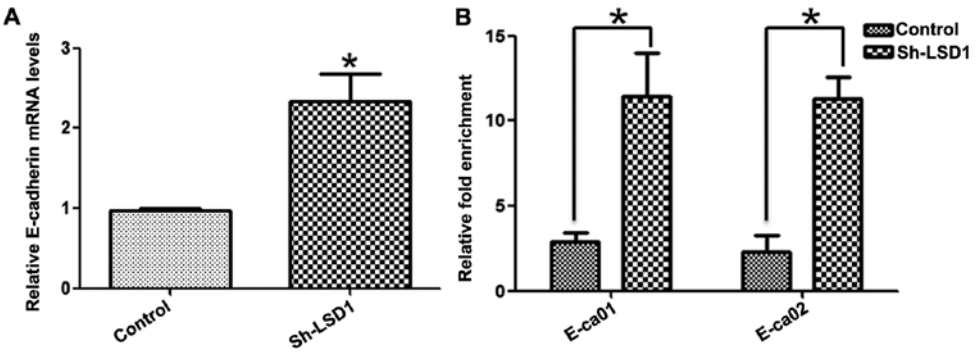

Figure 5. Knockdown of LSD1 decreases E-cadherin transcription and increases H3K4me2 levels at the E-cadherin promoter. (A) LSD1-KD HO8910 (sh-LSD1) cells were treated with 100 ng/ml doxorubicin hydrochloride (Dox) for 48 h, after which the E-cadherin mRNA levels were detected by qRT-PCR. (B) Cross-linked chromatin DNA from LSD1-KD HO8910 cells with or without 100 ng/ml Dox was immunoprecipitated with an anti-H3K4me2 antibody and analyzed by PCR with two primer sites of the E-cadherin promoter. *P<0.01 represents the relative fold enrichment in the LSD1-KD cells compared with the H08910 cells. This experiment was repeated at least three times.

EMT markers in the LSD1-KD and LSD1-OE HO8910 cells. We found that knockdown of LSD1 upregulated the expression of the epithelial marker E-cadherin and downregulated the expression of the mesenchymal markers N-cadherin, vimentin and MMP-2 (Fig. 4A). LSD1 knockdown also caused a decrease in the expression of the transcription factor Snail (Fig. 4A). Furthermore, inhibition of LSD1 induced an increase in E-cadherin expression and a decrease in the expression of N-cadherin, vimentin, MMP-2 and Snail in a dose-dependent manner (Fig. 4C). On the contrary, overexpression of LSD1 induced a decrease in E-cadherin expression, with a concomitant increase in the expression of N-cadherin, Vimentin, MMP-2 and Snail in the HO8910 cells (Fig. 4B).

LSD1 knockdown increases H3K4me2 levels at the E-cadherin promoter. Given that knockdown of LSD1 was accompanied by the upregulation of E-cadherin at the transcriptional level (Fig. 5A) and inhibition of migration of ovarian cancer cells (Fig. 2A and B), we speculated that LSD1 could enhance migration by downregulating E-cadherin expression via demethylation of H3K4me2, a major substrate of LSD1 in ovarian cancer cells (30). To confirm this speculation, ChIP assays were performed in the LSD1-KD HO8910 cells incubated with the anti-H3K4me2 antibody. Quantitative analysis indicated that the enrichment of H3K4me2 at the promoter of the E-cadherin gene was significantly higher in the LSD1-KD cells than that in the control cells (Fig. 5B). Collectively, our data revealed that the expression of LSD1 caused a decrease in H3K4me2 levels at the E-cadherin promoter, reduced E-cadherin expression, and consequently contributed to the migration of HO8910 cells.

Discussion

Ovarian cancer is the second most common malignant gynecologic tumor, and represents the leading cause of cancer-related death among women worldwide (1). The high mortality rate of ovarian cancer is caused by tumor metastasis, post-surgical recurrence, and late detection at advanced stages (3). Ovarian cancer is associated with multiple risk factors and is currently recognized as both a genetic and epigenetic disease (37,38). While the genetic changes in ovarian cancer have been extensively studied, the contribution of epigenetic alterations to ovarian cancer progression remains poorly understood. Histone methylation is a dynamic epigenetic process that has been found to be associated with cancer, including ovarian cancer (39). LSD1 is a well-characterized demethylase that can remove methyl groups from H3K4 (4). However, its role and underlying mechanisms in ovarian cancer are still unclear. In this study, we showed that LSD1 overexpression induced EMT, migration and invasion of HO8910 ovarian cancer cells. In contrast, silencing of LSD1 reversed these events in invasive

HO8910 cells. We also showed a mechanistic link between LSD1 and E-cadherin through LSD1-mediated regulation of H3K4me2, which subsequently leads to the downregulation of E-cadherin transcription.

Histone demethylases are epigenetic enzymes that can remove both repressive and activating histone marks. LSD1 family members are capable of removing the H3K4me2-activating marks and rendering them potential players in the downregulation of tumor suppressors (40,41). The putative role of LSD1 as an oncogene in cancer development is supported by the observation that LSD1 is highly expressed in ovarian cancer (13,14) and other malignant tumors (5-10). LSD1 is reported to play an important role in ovarian cancer cell proliferation via a Sox2-mediated mechanism (31). Our present study points to a novel function of LSD1 in ovarian cancer cell migration and invasion through regulation of EMT.

Recently, the regulation of epigenetic modification on EMT is a hot topic. Several studies have shown that histone modifications are involved in Snail-mediated transcriptional repression of E-cadherin. Peinado *et al* reported that Snail induces repressive histone modifications at the E-cadherin promoter through recruitment of histone deacetylases (HDACs) (42). Recent studies have demonstrated that Snail recruits LSD1 to the E-cadherin promoter to reduce E-cadherin expression by removing H3K4me2 (27,28). In this study, we found that modulation of LSD1 expression alters the methylation status of H3K4 at the E-cadherin promoter, which in turn transcriptionally regulates the expression of E-cadherin. Thus, we conclude that LSD1 transcriptionally downregulates E-cadherin expression via H3K4 demethylation, and consequently results in the increased migration and invasion of HO8910 cells.

Taking all these pieces of evidence together, we are able to show that knockdown of LSD1 impairs the migration and invasion of HO8910 cells by regulating EMT, while overexpression of LSD1 has a converse effect on cell migration. By demethylating H3K4me2 at the E-cadherin promoter, LSD1 downregulates the E-cadherin expression, and contributes to the metastasis of HO8910 cells. Our results suggest that LSD1 may be a potential therapeutic target for metastatic ovarian cancer.

Acknowledgements

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