Overexpression of lysine-specific demethylase 1 promotes androgen-independent transition of human prostate cancer LNCaP cells through activation of the AR signaling pathway and suppression of the p53 signaling pathway

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Abstract. Lysine-specific demethylase 1 (LSD1) is the first defined histone demethylase, and was found to be closely correlated with the development and progression of various types of cancers, including prostate cancer (PCa). Previous research suggests that LSD1 is closely related with cell proliferation, angiogenesis, migration and invasion in PCa. However, it remains to be elucidated whether LSD1 is correlated with androgen-independent (AI) transition of PCa under androgen-ablated conditions. The present study aimed to investigate the correlation of LSD1 expression with AI transition of human androgen-dependent PCa LNCaP cells. Our data showed that LSD1 was overexpressed in human PCa specimens and in AI PCa LNCaP-AI cells, which were established through a three-month continuous culture of LNCaP cells in androgen-deprived medium. Under androgen-deprived conditions, LNCaP-AI cells grew perfectly with less apoptosis and G_0/G_1 cell cycle arrest. Overexpression of LSD1 protected the LNCaP cells from androgen deprivation-induced apoptosis and G_0/G_1 arrest, while knockdown of LSD1 drove LNCaP-AI cells into a higher rate of apoptosis and G_0/G_1 arrest. Furthermore, LSD1 was found to regulate the androgen receptor (AR) and p53 signaling pathways via demethylation, subsequently influencing apoptosis and cell cycle progression. These findings revealed that overexpression of LSD1 promoted AI transition of PCa LNCaP cells under androgen-ablated conditions via activation of the AR signaling pathway and suppression of the p53 signaling pathway.

Introduction

Worldwide, prostate cancer (PCa) is the second most frequently diagnosed cancer in men and the fifth leading cause of cancer-related death (1). Recently, the incidence of PCa in China has been substantially increasing with implementation of comprehensive screening programs (2). For the majority of PCa patients, endocrine therapy is effective at first, but almost all patients undergo progression from androgen-dependent prostate cancer to androgen-independent prostate cancer (AIPC) following a median period of 14-30 months. To date, the specific mechanisms involved in androgen-independent (AI) transition have not been entirely clarified.

Lysine-specific demethylase 1 (LSD1) is the first defined histone demethylase, and specifically demethylates histone H3 at the lysine K4 locus and remodels chromatin to regulate gene expression (3). LSD1 protein was reported to interact with androgen receptor (AR) protein and demethylate histone H3 at lysine 9 (H3-K9) to repress the AR target gene (4,5). In addition, LSD1 protein interacts with and demethylates p53 protein, a non-histone protein, at lysine K370 to repress its function (6,7). The biological effects of LSD1 have been studied most extensively, and evidence demonstrates that LSD1 is aberrantly overexpressed in various types of human cancers, such as breast, lung, colorectal, bladder and ovarian cancer, and plays crucial roles in tumorigenesis and progression (8-13).

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LSD1 is significantly upregulated in PCa and may serve as a predictive biomarker of aggressive PCa (14-16). Accumulated data imply that LSD1 is closely correlated with cell proliferation, angiogenesis, migration and invasion in PCa (17-19). However, the correlation of LSD1 with AI transition of PCa under androgen-ablated conditions has rarely been reported.

AR is thought to play a critical role in the development of AIPC via various mechanisms, including AR amplification, mutation and activation via ligand-independent modifications, increased AR sensitivity to low-level androgen, and bypass of intact AR pathways (20-23). The transcription factor p53 functions as a tumor-suppressor gene, and is activated by DNA damage, deficiency of nutrients or growth factors, and induces apoptosis and cell cycle arrest (24). p53 gene mutation is quite common in human cancers, such as late-stage PCa (25,26). Reportedly, LSD1 interacts with AR and p53 and regulates their biological functions through post-translational demethylation (4,6).

The present study aimed to ascertain whether LSD1 is involved in AI transition of human androgen-dependent prostate cancer LNCaP cells. Our data revealed that overexpression of LSD1 'rescued' LNCaP cells from androgen ablation-induced apoptosis and G_0/G_1 cell cycle arrest and promoted androgen-independent transition via activation of the AR signaling pathway and suppression of the p53 signaling pathway.

Materials and methods

Patients and specimens. From December 2010 to October 2013, prostate cancer tissues and paired adjacent non-tumor tissues were collected from 20 PCa patients undergoing radical prostatectomy at the Department of Urology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. Fresh tissues were used to isolate total RNA for reverse-transcription quantitative-polymerase chain reaction (RT-qPCR) and total protein for western blotting. The diagnoses of PCa were confirmed by histopathological examination. The study involving the use of human prostate cancer specimens was approved by the Institutional Review Board of Tongji Medical College of Huazhong University of Science and Technology. Written informed consents was obtained from each participant.

Cell culture and establishment of the LNCaP-AI cell model. The human prostate cancer LNCaP cell line was purchased from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). LNCaP cells were cultured in Phenol Red-containing RPMI-1640 medium containing 10% fetal bovine serum (FBS) (both from Gibco, Carlsbad, CA, USA) at 37°C in a 5% CO_2 incubator. To obtain AI cells, the LNCaP cells were continuously cultured in Phenol Red-negative RPMI-1640 medium (Gibco) supplemented with 10% charcoal-stripped FBS (CS-FBS; Biowest, Nuaillé, France) for three months (27). During the three-month period, morphological changes in the LNCaP cells were dynamically observed.

Cell transfection. Overexpression plasmids for LSD1 and NC were constructed by Shanghai GeneChem Co. (Shanghai,

China). sh-LSD1 and sh-NC were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). LNCaP and LNCaP-AI cells ($8x10^5$/well) were seeded in 6-well plates and maintained in antibiotic-free complete culture medium for 24 h. At transfection, the medium was replaced with 1,500 μl of antibiotic-free and FBS-free medium per well. Lipofectamine 2000 reagent (6 μl) (Invitrogen, Carlsbad, CA, USA) and 3 μg of plasmid were diluted in 250 μl Opti-MEM Reduced Serum Medium (Gibco), respectively, and incubated for 5 min at room temperature. Diluted plasmid was added into the diluted Lipofectamine 2000 reagent and incubated for 20 min at room temperature. A plasmid-lipid complex (500 μl) was added to each well. Stably transfected cells were selected by G418 (EMD Chemicals, Inc., San Diego, CA, USA) or puromycin (Santa Cruz Biotechnology).

Cell Counting Kit-8 (CCK-8). Cells ($1x10^3$/well) were seeded into 96-well plates and cultured in Phenol Red-containing medium with 10% FBS or Phenol Red-negative medium with 10% CS-FBS for 0, 2, 4, 6 and 8 days, respectively. CCK-8 solution (10 μl) (Dojindo, Kumamoto, Japan) was added into each well and incubated for 2.5 h. Optical density (OD) values were measured at 450 nm on a microplate reader (Tecan, Männedorf, Switzerland).

Enzyme-linked immunosorbent assay (ELISA). LNCaP-AI and LNCaP cells ($1x10^4$cells/well) were plated into 24-well plates and cultured in Phenol Red-containing medium with 10% FBS or Phenol Red-negative medium with 10% CS-FBS for 2, 4, 6 and 8 days, respectively. Culture supernatants were harvested, and the protein levels of prostate-specific antigen (PSA) were determined using the Human PSA Immunoassay (R&D, Minneapolis, MN, USA) according to the manufacturer's instructions.

Cell apoptosis analysis via flow cytometry. Cell apoptosis was detected using the Annexin V-FITC apoptosis detection kit (Beyotime Biotechnology, Jiangsu, China). Cells ($5x10^5$ cells/flask) were seeded into a 25 cm^2 culture flask and maintained in Phenol Red-negative medium with 10% CS-FBS for 96 h. Cells were collected, suspended in Annexin V-FITC binding buffer, incubated with Annexin V-FITC and PI for 20 min at room temperature in the dark and detected by flow cytometry.

Cell cycle analysis by flow cytometry. The cell cycle was analyzed using a cell cycle and apoptosis analysis kit (Beyotime). Cells ($5x10^5$ cells/flask) were seeded into a 25 cm^2 culture flask and maintained in Phenol Red-negative medium with 10% CS-FBS for 48 h. Cells were collected, fixed with ice-cold 70% ethanol for 24 h at 4°C, washed, stained with PI staining solution for 30 min at room temperature in dark, and detected via flow cytometry.

RNA extraction and RT-qPCR. Total RNA was isolated from tissues or cells with RNAiso Plus (Takara Biotechnology Co., Ltd., Dalian, China) following the manufacturer's instructions. Total RNA was retro-transcribed into cDNA using the RevertAid™ First Strand cDNA synthesis kit (Thermo Fisher Scientific Inc., Beijing, China) following the manufacturer's
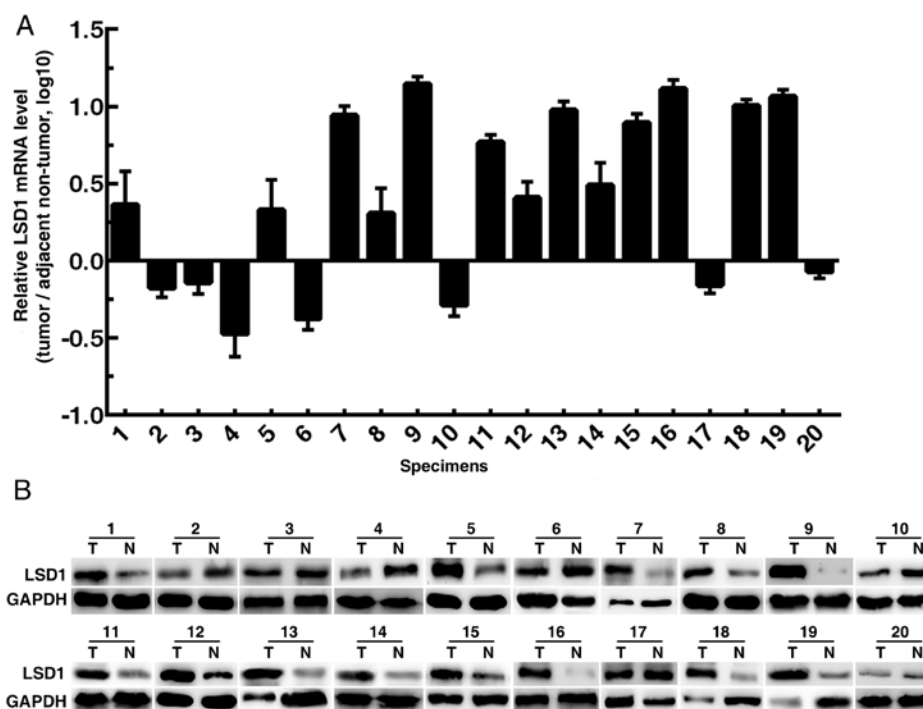
Figure 1. Expression of lysine-specific demethylase 1 (LSD1) in human prostate cancer specimens (tumor tissues and paired adjacent non-tumor tissues). (A) Reverse-transcription quantitative polymerase chain reaction (RT-qPCR). (B) Western blotting.

protocols. RT-qPCR was carried out with Thermo Scientific Maxima SYBR-Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific Inc.) on the StepOne™ real-time PCR system (Life Technologies, Grand Island, NY, USA). The primer pairs were searched from PrimerBank and synthesized by Life Technologies. PCR was performed as follows: initial denaturation for 10 min at 95°C, and then amplification of 40 cycles at 95°C for 15 sec and 60°C for 60 sec. GAPDH was used as endogenous control. The results were analyzed by the $2^{-\Delta\Delta CT}$ method. The primer pairs were as follows: LSD1, 5'-TGACCG GATGACTTCTCAAGA-3' (sense) and 5'-GTTGGAGAGTA GCCTCAAATGTC-3' (antisense); AR, 5'-CCAGGGACCAT GTTTTGCC-3' (sense) and 5'-TCTGGGGTGGAAAGTAAT AGTCA-3' (antisense); p53, 5'-TTTGCGTGTGGAGTATTTG GAT-3' (sense) and 5'-CAACCTCAGGCGGCTCATA-3' (antisense); GAPDH, 5'-AAGGTGAAGGTCGGAGTCAAC-3' (sense) and 5'-GGGGTCATTGATGGCAACAATA-3' (antisense).

Western blot analysis. Total protein was extracted using cell lysis buffer (Beyotime). Proteins were separated on SDS-PAGE gels and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk, the membranes were incubated with the primary antibodies at 4°C overnight, followed by incubation with peroxidase-conjugated secondary antibodies (Proteintech, Wuhan, China). Protein bands were developed with BeyoECL Plus (Beyotime) on a Kodak Image Station 4000MM (Eastman Kodak Company, Rochester, NY, USA). The primary antibodies against rabbit polyclonal LSD1 (1:1,000; Abcam, Cambridge, MA, USA), mouse monoclonal AR (1:1,000), rabbit polyclonal p53 (1:500) (both from Santa Cruz), rabbit polyclonal H3 mono-methyl K9 (1:500) and mouse monoclonal H3 di-methyl K9 (1:500)

(both from Abcam), rabbit polyclonal p53 di-methyl K370 (1:500; Ameritech Biomedicines, Houston, TX, USA), rabbit monoclonal caspase-9 (1:500; Beyotime), mouse monoclonal caspase-8 (1:500; Proteintech), rabbit polyclonal caspase-3 (1:500; Beyotime), rabbit polyclonal Bax (1:1,000), rabbit polyclonal PUMA (1:1,000), rabbit polyclonal Bcl-2 (1:1,000; all from Proteintech), rabbit monoclonal Bcl-xL (1:1,000; Beyotime), rabbit polyclonal cyclin A (1:1,000; Santa Cruz), rabbit polyclonal cyclin E1 (1:1,000; Proteintech), mouse monoclonal CDK2 (1:1,000; Santa Cruz), mouse monoclonal cyclin D1 (1:1,000; Proteintech), rabbit polyclonal CDK4 (1:1,000) and rabbit polyclonal p21 (1:1000) (both from Santa Cruz) and mouse monoclonal GAPDH (1:4,000; Proteintech) were used. GAPDH was used as the loading control.

Co-immunoprecipitation (Co-IP). Co-IP was performed using Pierce Co-immunoprecipitation kit (Pierce Biotechnology, Rockford, IL, USA) following the manufacturer's protocols. Antibodies (LSD1, AR and p53) were immobilized onto the resin in different spin columns, respectively. Cells were lysed with ice-cold Pierce IP lysis. Each lysate was pre-cleared using Pierce control Agarose Resin slurry at 4°C for 1 h with gentle end-over-end mixing. Each sample of total protein was added into a spin column containing the antibody-coupled resin, and incubated with gentle rocking at 4°C overnight, followed by centrifugation. Elution buffer were added, incubated at room temperature for 5 min and centrifuged. Flow-throughs were collected for SDS-PAGE analysis.

Statistical analysis. All experiments were repeated twice. Data analysis was carried out with the Statistical Package for the Social Sciences (SPSS), version 17.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation
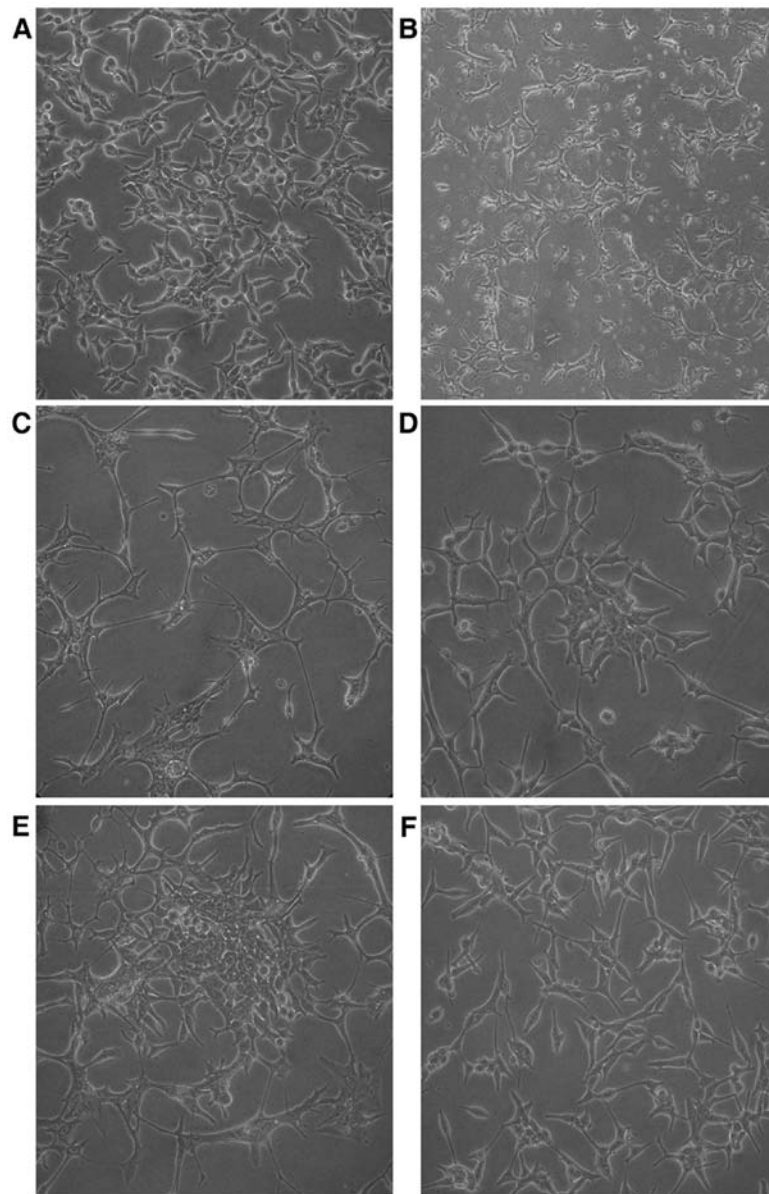
Figure 2. Establishment of the LNCaP-AI cell model. (A and B) Original magnification, x100. (C-F) Original magnification, x200.

and P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of LSD1 in human PCa specimens. Twenty pairs of PCa specimens (tumor tissues and adjacent non-tumor tissues) were collected to perform RT-qPCR and western blotting. The results showed that LSD1 expression in tumor tissues was upregulated in 65% (13/20) of the specimens (Fig. 1).

Establishment of the LNCaP-AI cell model. In androgen-containing medium, the LNCaP cells grew well and showed an epithelial morphology. The cell body was large and cell processes were small and short (Fig. 2A). In androgen-deprived medium, the LNCaP cells barely proliferated, and showed a neuroendocrine-like morphology for a long period. The cell body turned small, and cells were connected with each other via increased and elongated processes (Fig. 2B and C). After 2 months, the LNCaP cells began to grow and form cell colonies slowly, and elimination of the neuroendocrine-like state occurred gradually (Fig. 2D and E). At the end of the third month, cells proliferated rapidly and the neuroendocrine-like state was not observed (Fig. 2F). At this point, the obtained cells were androgen-independent and were named as LNCaP-AI cells.

Biological characteristics of the LNCaP and LNCaP-AI cells. CCK-8 assay showed that proliferation of the LNCaP-AI cells was unimpeded, while growth of the parental LNCaP cells was obviously inhibited in the androgen-deprived medium. Both LNCaP-AI and LNCaP cells proliferated rapidly in the androgen-containing medium (Fig. 3A). In line with this, the PSA concentration did not alter distinctly with time in the LNCaP cell supernatant in androgen-deprived medium, but increased in the LNCaP-AI cell supernatant. Meanwhile, PSA concentrations in the LNCaP-AI and LNCaP cell supernatants were both elevated continuously in the androgen-containing-
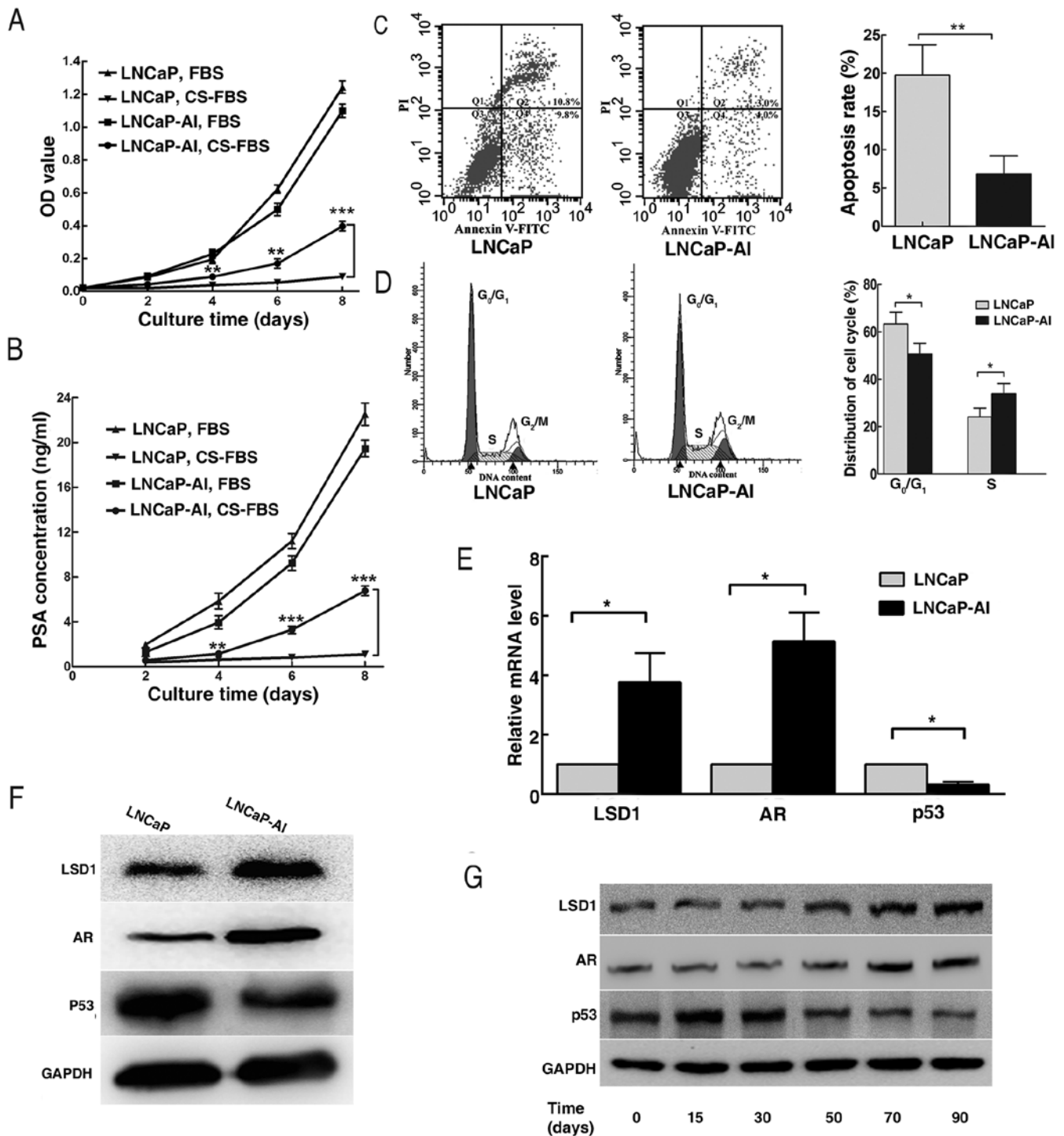
Figure 3. Biological characteristics of the LNCaP and LNCaP-AI cells. (A) Cell proliferation. (B) PSA secretion. (C and D) Cell apoptosis and cell cycle analyses. (E-G) Western blot analysis. ***P<0.001, **P<0.01, *P<0.05.

medium (Fig. 3B). Flow cytometry revealed that the LNCaP cells showed a high rate of apoptosis and G_0/G_1 cell cycle arrest in androgen-deprived medium, compared with the LNCaP-AI cells (Fig. 3C and D). RT-qPCR and western blotting analysis indicated that expression levels of LSD1 and AR in the LNCaP-AI cells were upregulated, while expression of p53 was downregulated (Fig. 3E and F). Moreover, expression levels of LSD1, AR and p53 in the LNCaP cells at the indicated time points during androgen ablation displayed the same trends as described above (Fig. 3G).

Overexpression of LSD1 promotes LNCaP cell survival under androgen-deprived conditions. LSD1 was overexpressed in the LNCaP cells and knocked down in the LNCaP-AI cells by plasmid transfection. Overexpression and knockdown efficiencies of LSD1 were verified by western blotting (Fig. 4A). CCK-8 and flow cytometry revealed that the LNCaP-LSD1 cells displayed more rapid proliferation, a higher PSA concentration in the culture supernatant and a lower apoptosis rate and G_0/G_1 phase arrest in androgen-ablated medium, in contrast to the LNCaP-NC cells, while LNCaP-AI-sh-LSD1
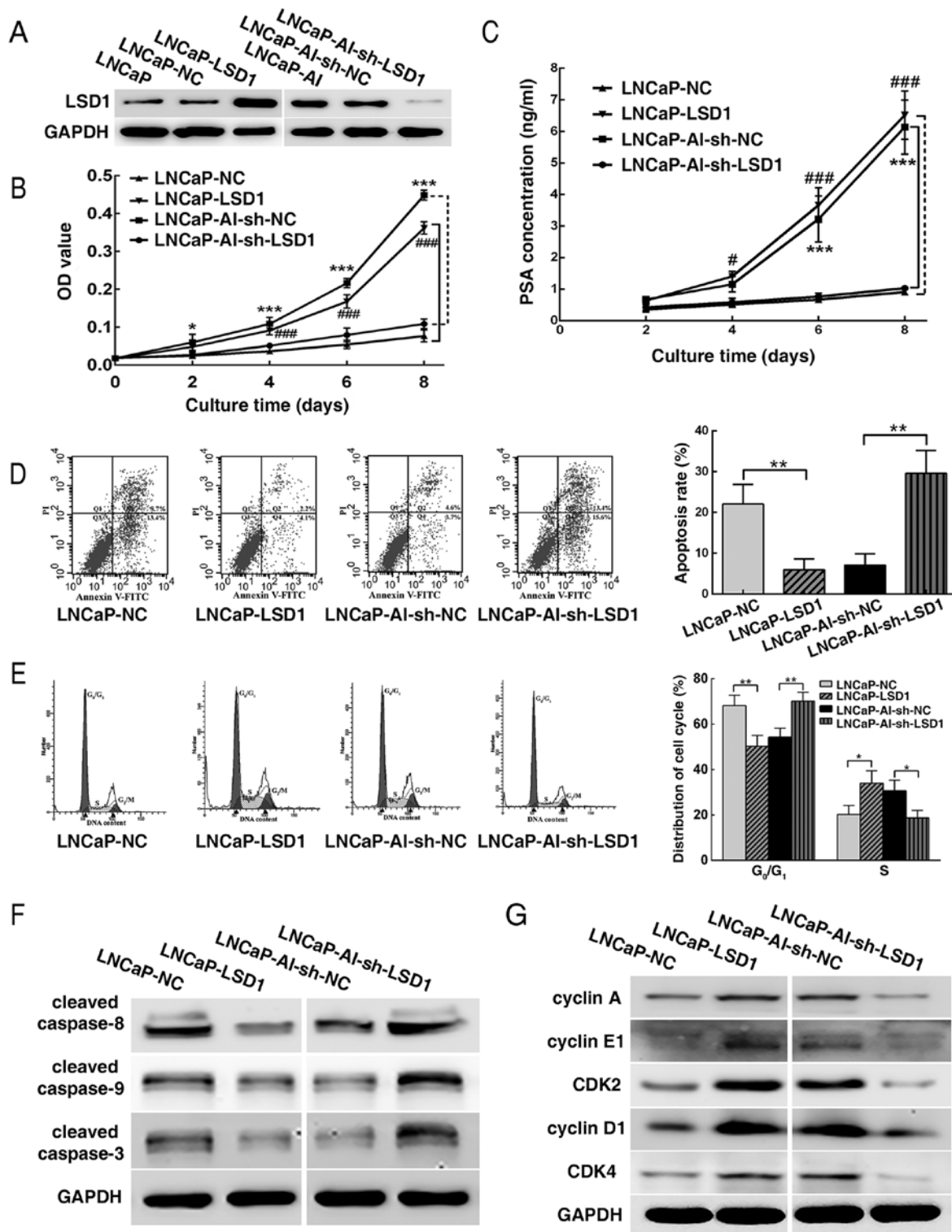
Figure 4. Overexpression of LSD1 promotes LNCaP cell survival under androgen-deprived conditions. (A) Verification of LSD1 transfection. (B) Cell proliferation. (C) PSA secretion. (D and E) Cell apoptosis and cell cycle analyses. (F and G) Western blot analysis. ***,###$P<0.001$, **,##$P<0.01$, *,#$P<0.05$.

cells showed a slower growth, lower PSA concentration in the culture supernatant and a higher apoptosis rate and G_0/G_1 arrest, compared with the LNCaP-AI-sh-NC cells (Fig. 4B-E). Furthermore, western blot analysis revealed that cleaved caspase-8, cleaved caspase-9 and cleaved caspase-3 were downregulated in the LNCaP-LSD1 cells when compared to the LNCaP-NC cells. Expectedly, the

opposite results occurred in the LNCaP-AI-sh-LSD1 cells vs. the LNCaP-AI-sh-NC cells (Fig. 4F). In a similar manner, expression levels of cyclin A, cyclin E1, CDK2, cyclin D1 and CDK4 were upregulated in the LNCaP-LSD1 cells vs. the LNCaP-NC cells, while the opposite results appeared in the LNCaP-AI-sh-LSD1 cells vs. the LNCaP-AI-sh-NC cells (Fig. 4G).
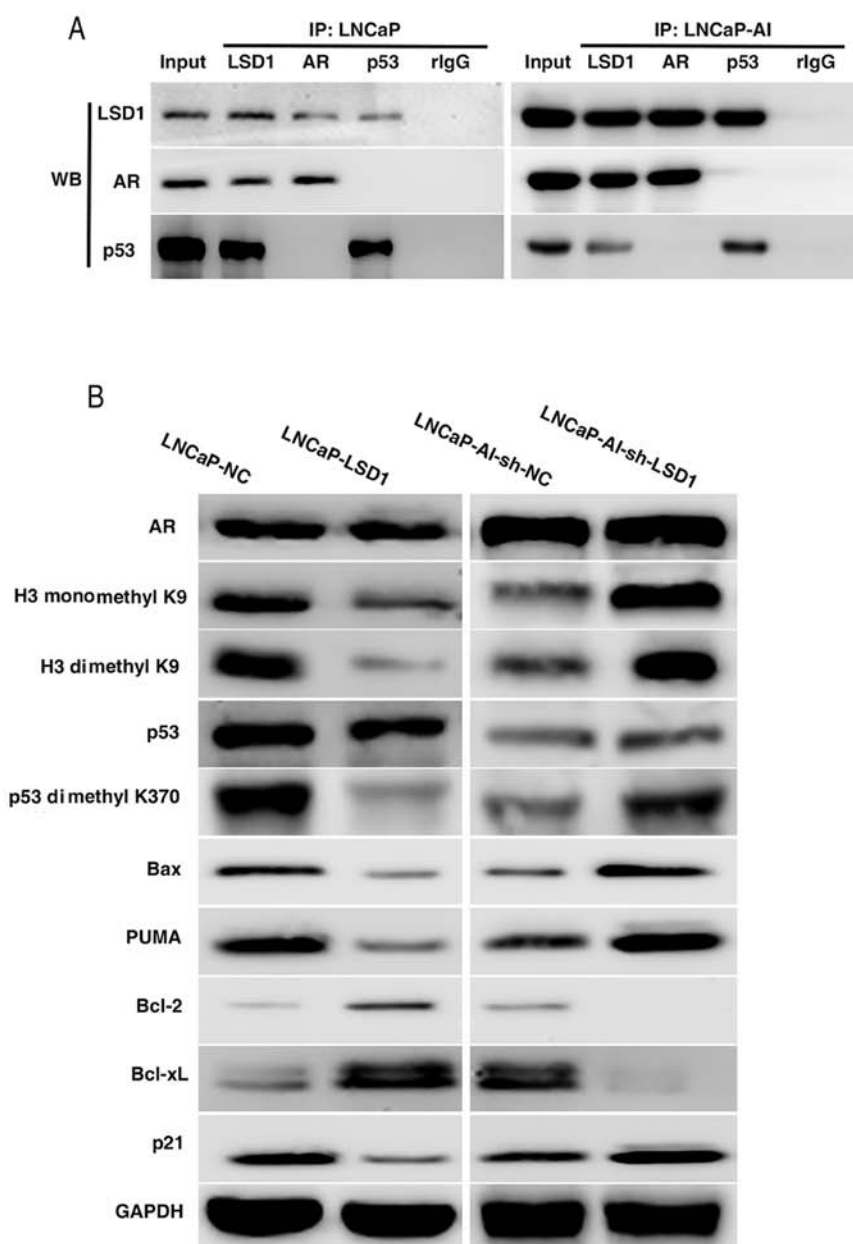
Figure 5. Overexpression of LSD1 rescues LNCaP cells from androgen deprivation through activation of the AR signaling pathway and suppression of the p53 signaling pathway. (A) Co-immunoprecipitation. (B) Western blotting.

Overexpression of LSD1 activates AR and suppresses the p53 signaling pathway through demethylation. Co-immunoprecipitation (Co-IP) assay was carried out to ascertain whether LSD1 protein interacts with AR and p53 proteins in the LNCaP and LNCaP-AI cells. Western blot analysis indicated that AR protein and p53 protein were detected in the immunoprecipitated proteins by LSD1 antibody. LSD1 protein was detected in the immunoprecipitated proteins by the AR antibody or p53 antibody (Fig. 5A), which confirmed that LSD1 protein interacted with the AR and p53 proteins.

When LSD1 was overexpressed in the LNCaP cells or knocked down in the LNCaP-AI cells, the protein expression levels of AR and p53 were not obviously changed, while the methylation of histone H3-K9 and protein p53 K370 was distinctly altered (Fig. 5B). Mono- and di-methylation of H3-K9 and di-methylation of p53 K370 were decreased with

LSD1 overexpression and were increased following LSD1 knockdown. p53 transactivates pro-apoptotic genes Bax and PUMA and cyclin-dependent kinase inhibitor p21, and transcriptionally represses anti-apoptotic genes Bcl-2 and Bcl-xL. Western blotting revealed that Bax, PUMA and p21 were downregulated, while Bcl-2 and Bcl-xL were upregulated, when dimethylation of p53 K370 was decreased in the LNCaP-LSD1 cells vs. the LNCaP-NC cells. Expectedly, opposite results were noted in the LNCaP-AI-sh-LSD1 cells vs. the LNCaP-AI-sh-NC cells (Fig. 5B).

Discussion

In the present study, we verified that the expression of LSD1 was upregulated in human PCa specimens, which implied the oncogenic function of LSD1. Reportedly, overexpression of

LSD1 is involved in tumorigenesis, progression, relapse and poor prognosis in PCa (14,15,17,28).

Development of AI growth is a major obstacle to the treatment of PCa. An ideal cell model pair is deeply desired to better understand the molecular mechanisms of AI transition. The LNCaP-AI cell line was generated by a 3-month culture of androgen-dependent LNCaP cells in androgen-deprived medium (29,30) and was defined by the ability of proliferating under androgen-ablated conditions. The pair of cell lines was perfectly suitable for our investigation. During the androgen-deprived process, LNCaP cells showed an obvious neuroendocrine-like morphology (31), which possibly rescued PCa cells from androgen ablation (32-34).

We compared the biological behaviors of the parental LNCaP and LNCaP-AI cells and it was shown that LNCaP-AI cells proliferated well and restored the secretory capacity of PSA in the absence of androgen. This phenomenon, in accordance with the PSA levels in clinical patients with AIPC, validated the success of the AIPC cell model establishment. Notably, LNCaP-AI and LNCaP cells proliferated faster and produced a higher PSA concentration in the androgen-containing environment, which showed that LNCaP-AI cells remained androgen-responsive. Based on this finding, the priority in clinical management of AIPC patients is to assure effective androgen ablation. In addition, our study revealed that LNCaP-AI cells had a lower apoptosis rate and G_0/G_1 arrest under androgen deprivation. Reduction in apoptosis and cell cycle dysfunction play vital roles in cancer development and progression, which might assist AI transition of PCa during androgen ablation.

AR facilitates androgen-independent cellular proliferation and cell cycle progression and contributes to the development of AIPC (35,36). p53 affects apoptosis and cell cycle progression, and its frequent disability may result in tumor development and the failure of anti-neoplastic therapies (37,38). Our data showed overexpression of LSD1 and AR and downregulation of p53 in the LNCaP-AI cell line and in its established process.

Following overexpression of LSD1, LNCaP cells manifested rapid proliferation, increased PSA production and less apoptosis and G_0/G_1 arrest under androgen ablation, along with downregulation of cleaved caspases and cyclins/CDKs; knockdown of LSD1 inhibited growth and PSA secretion in the LNCaP-AI cells and induced enhanced apoptosis and cell cycle arrest at G_0/G_1 phase in the absence of androgen, accompanied with upregulation of cleaved caspases and cyclins/CDKs. Co-IP assay revealed that LSD1 protein interacted with AR and p53 proteins in the LNCaP and LNCaP-AI cells, in accordance with previous reports (4,6). However, LSD1 overexpression and knockdown did not obviously change the protein expression levels of AR and p53, but the methylation of histone H3-K9 and protein p53 K370. Overexpression of LSD1 reduced mono- and di-methylation of H3-K9 and di-methylation of p53 K370 and knockdown of LSD1 increased these methylations. Demethylation of mono- and di-methylated residues of H3-K9 is an important marker of AR-activated gene expression (4). Overexpression of LSD1 increased PSA secretion in the LNCaP cells, while knockdown of LSD1 reduced PSA production in the LNCaP-AI cells, which implied that LSD1 regulated the expression of the AR target gene through histone demethylation. Demethylation

of di-methylated residues of p53 K370 negatively regulates the interaction of p53 and co-activator 53BP1 and represses the transcriptional activity of p53 (6).

Apoptosis is an active cell suicide process and maintains cellular homeostasis. However, cancer cells can override apoptosis through upregulating anti-apoptotic machinery and/or downregulating the pro-apoptotic program (39). Previous research has revealed that tumor suppressor and transcription factor p53 regulates apoptosis through transactivation of Bax and PUMA and transcriptional repression of Bcl-2 and Bcl-xL (24,40,41). Control of the cell cycle monitors cell growth and DNA integrity, but uncontrolled cell cycle progression can contribute to genomic instability and oncogenesis (42). p53 transcriptionally controls the p21 gene, which acts as a cyclin-CDK inhibitor and takes charge of negative regulation of the cell cycle (43,44). Our data revealed that overexpression of LSD1 in LNCaP cells resulted in decreased protein levels of Bax, PUMA and p21 and increased protein levels of Bcl-2 and Bcl-xL, while knockdown of LSD1 in LNCaP-AI cells increased the expression levels of Bax, PUMA and p21 and reduced the expression levels of Bcl-2 and Bcl-xL. Taken together, these findings indicate that LSD1 regulates the activity of p53 through demethylation, and thus regulates apoptosis and the cell cycle.

In conclusion, LSD1 is upregulated in human prostate cancer and plays an oncogenic role. During androgen ablation, LSD1 may contribute to AI transition of prostate cancer LNCaP cells through activation of the AR signaling pathway and suppression of the p53 signaling pathway. Our findings may elucidate another mechanism involved in AIPC development.

Acknowledgements

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