

## Original Article

# Repressive effects of LSD1 inhibitor pargyline on cellular proliferation, motility and EMT process of prostate cancer in vitro

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**Abstract:** Objective: To elucidate the effect of lysine-specific demethylase 1 (LSD1) inhibitor pargyline on cellular proliferation, migration and the process of epithelial-mesenchymal-transition (EMT) in human prostate cancer *in vitro*. Methods: The roles of LSD1 inhibitor pargyline in cellular proliferation in prostate cancer cells *in vitro* were evaluated by CCK8, colony formation assay, and flow cytometry. Wound healing and transwell assay were used to evaluate the motility ability. Then  $\beta$ -actin and core genes of EMT (E-cadherin, N-cadherin, and Vimentin) were measured by western blotting and RT-qPCR to demonstrate the effects on EMT process. Clinical data was obtained from the online data sites (GEO, STRING). Results: Compared with the control group, treatment with pargyline inhibited the cell viability and proliferation of DU145 and PC-3 cells. Treated with 3 mM pargyline for 48 h, the cell apoptosis significantly increased and the cell cycle was arrested. For cellular motility, the wound closure was delayed and the passed cells in transwell migration assay decreased. In addition, the process of EMT was blocked, resulting in up-regulated expression of E-cadherin and down-regulated N-cadherin and Vimentin in both cell lines. Conclusions: LSD1 inhibitor alleviated the proliferation, migration, and EMT process in human androgen-independent prostate cancer *in vitro*.

**Keywords:** Epithelial-mesenchymal-transition, lysine-specific demethylase 1, pargyline, prostate cancer, tumor-suppressor

## Introduction

Prostate cancer (PCa) was considered as the most prevalently diagnosed and estimated cancers occurred among males in western countries. In American, it had the most estimated new cases and was expected as the 2nd leading cause of cancer-related death for men [1]. In Europe, PCa outnumbered lung and colorectal carcinoma to become the most common solid neoplasm, with an incidence rate of 214 cases per 1000 men [2]. In China, compared with the data in 1990, cancer incidence and mortality rates of PCa appeared exponential growth in 2013. Approximately 81,400 new cases and 17,800 cancer deaths were projected to occur in 2013, respectively increasing 490.27% over 13,800 new cases while 206.86% over 5,800 deaths caused by PCa in 1990 [3]. The mechanism of tumor formation, development in PCa still remained largely elusive. The activation of androgen receptor

(AR), as one of the hormone related nuclear factors, was critical for prostate cancer cell progression and metastasis [4]. In the different process of PCa, it's common with the presence of abnormal activation or increased expression of AR stimulated by androgen, followed by abnormally active transcription of downstream genes. Accordingly, AR was considered as a marker of prostate cancer development and targeting AR remained an important therapeutic approach [5]. Recently, screening for AR-interacting proteins revealed overexpression of several AR-associated coactivators were essential for AR activation during disease progression [6, 7]. Identifying such coactivators and the stimuli signaling pathways in prostate cancer biology is a novel method to explore potential therapeutic target [8].

Lysine-specific demethylase 1 (LSD1, also known as KMDA1 or BHC110) was investigated to co-localize with the AR in both of normal

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human prostate and prostate cancer [9]. LSD1 interacted with AR via a ligand-dependent manner *in vitro* and *in vivo*, mediating AR-dependent transcription. Meanwhile, LSD1 was regarded as the first lysine demethylation to mediate the expression of genes. In eukaryotes, post-translation regulation, as one of the unique methods in eukaryotes, were investigated to contribute to the tumorigenesis, tumor progress and metastasis [10]. LSD1 moderated both gene activation and repression by demethylation at distinct lysine or threonine residues in histone H3 during AR-dependent gene expression [11]. LSD1 relieved repressive histone marks by demethylation of histone H3 at lysine 9 (H3-K9), leading to de-repression of AR target genes [12]. Meanwhile, LSD1 had also been found with demethylation effects on other epigenetic regulatory proteins such as DNA (cytosine-5)-methyltransferase 1 (DNMT1) [13]. Although the moderation network and mechanism remained largely unclear. LSD1 inhibitors probably offered a novel and potential alternative treatment for PCa.

Epithelial-mesenchymal transition (EMT) was regarded as a key regulator of metastasis in some cancers by conferring an invasive phenotype [14]. Various EMT-associated gene expression profiles were apparent and depended on cell and tissue type, and on the degree of progression towards mesenchymal differentiation. A core hallmark of EMT was the downregulation of E-cadherin (CDH1) to destabilize adherens junction [15]. In addition, down-expression of E-cadherin in combination with up-expression of N-cadherin (CDH2) resulted in 'cadherin switch', leading to weaker cell adhesion [16, 17]. Induction of vimentin concomitant with suppression of E-cadherin expression was also a biochemical hallmark of EMT [18].

We have proved the correlation between the expression of E-cadherin and LSD1, and the down-expression of E-cadherin negatively related with Gleason score and metastasis [12]. In this study, we observed LSD1 inhibitor pargyline had repressive effects on Du145 and PC-3 cell line. Pargyline could inhibit cellular proliferation, motility and EMT process of human androgen-independent prostate cancer *in vitro*.

### Material and methods

#### Cell culture

Human prostate cancer DU145 and PC-3 cell lines were purchased from the Cell Bank of the

Chinese Academy of Sciences (Beijing, China) and were cultured in RPMI-1640 medium supplemented with 10% FBS (Sijiqing, China), and 1% penicillin/streptomycin (Invitrogen) at 37°C. Cells were seeded into plates for 24 hours, then were received treatment with pargyline (Sigma, USA) at 0-3 mM for 24, 48 and 72 hours respectively.

#### Cell viability assay and colony formation assay

The effect of Pargyline on the cell viability of DU145 and PC-3 cells was performed using Cell Counting kit-8 (CCK8). Briefly, cells were seeded into a 96-well plate at the density of 800 cells per well and were treated with pargyline at 0-3 mM for 24, 48 and 72 hours. After treatment with pargyline, the cells were incubated with 10 $\mu$ L CCK8 for 1 hour at 37°C. Cell viability was measured at a wavelength of 450 nm. Meanwhile, the experiments were undertaken for screening a proper concentration and duration. For colony formation assay, PC-3 and DU145 were seeded at a density of 1000 cells per well and were allowed to grow undisturbed for 10 days. Cells were stained with crystal violet on the plates and counted.

#### Flow cytometric analysis

Prostate cancer cells ( $2.5 \times 10^4$ ) were planted in 6-well plants. We used the flow cytometer to analyze apoptosis and cell cycle distribution. Briefly, DU145 and PC-3 cells were treated with pargyline at the concentration of 3 mM for 48 hours. The effect of pargyline on cell cycle of DU145 and PC-3 cells were determined using propidium iodide (PI) as DNA stain. Cells were trypsinized, washed with PBS, and fixed by 75% ethanol at -20°C overnight. The fixed cells were stained using PI. A total number of  $1 \times 10^4$  cells was collected for cell cycle analysis using the flow cytometer.

#### Wound-healing assay

Cells at the concentration of  $2 \times 10^4$  per well were seeded in 6-well plates and incubated until near confluent monolayers (over 80%) in RPMI 1640 supplemented with 10% serum before the treatment. After serum starvation for 24 hours, cell monolayers were wounded by scraping with an aseptic 200- $\mu$ l pipette tip as the 0-h time point. The cells were incubated in medium with 10% serum and taken photos at different time points (24, 48 hours). Before photographing, the cells were washed twice

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with PBS and supplemented with the fresh serum-containing medium. The rate of wound healing was determined by relative width compared with the original wound (Relative width =  $W_{24H}/W_{0H}$ ;  $W_{0H}$ , wound width at 0H,  $W_{24H}$ , wound width at 24 H).

### *Transwell assay*

For migration assays, the transwell inserts with an 8  $\mu$ m pore size (BD Biosciences) for 24-well plates were used. DU145 and PC-3 cells were seeded in the upper chamber at  $1 \times 10^4$  cells per well in RPMI 1640 serum-free medium, while serum containing medium was added in the bottom chamber. After 24 hours, cells through the pore was fixed with 4% paraformaldehyde following removing cells on the upper surface of the filter by a cotton swab. Then the upper chamber was stained with 10% crystal violet for 30 minutes before cell counting by using the inverting microscope. Randomly five fields of each chamber were selected (Cells which did not finish migrating through the pore in regular shape were not included).

### *Western-blotting analyzing*

The protein expression of  $\beta$ -actin, E-cadherin, N-cadherin, and Vimentin were examined. Briefly, DU145 and PC-3 cells were lysed with RIPA containing 1% PFMA for 15 min at 4°C and transferred to the centrifuge at 12000 g for 10 min. The centrifugal supernatant was measured by bicinchoninic acid assay (BCA) and denatured for 5 minutes in boiled water. The protein samples were electrophoresed on 8%-12% SDS-PAGE gel (30-50  $\mu$ g/lane) and transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA) at 100 V for 120 minutes at 4°C. The membranes were blocked with 5% nonfat milk in TBST buffer (10 mmol/L Tris-HCl, 0.15 mol/L NaCl, 0.05% Tween 20, pH 7.2) for 2 hours then probed with primary antibody against E-cadherin (1:1000 dilution; Cell Signalling, USA), and N-cadherin (1:1000 dilution; Cell Signalling, USA), Vimentin (1:1000 dilution; Cell Signalling, USA) overnight at 4°C and then blotted with the respective secondary antibody. The proteins were detected using an enhanced chemiluminescence system (ECL kit, Pierce Biotechnology, Beijing, China) and captured on light-sensitive X-ray film (Kodak, Sh-

anghai, China). Optical densities were detected by using ImageJ software.

### *RNA isolation and real-time quantitative PCR*

To determine the mRNA levels of E-cadherin, N-cadherin, and vimentin, we used a quantitative real-time PCR (RT-qPCR) assay with SYBR Green PCR Core Reagents kit. After treatment with pargyline at different concentrations (0 mM, 1 mM, and 3 mM) for 48 hours, DU145 and PC-3 cells were harvested. The total RNA was isolated from cells using the TRIZOL reagent (Invitrogen, USA) following the manufacturer's protocol. The concentration and purity of the RNA were examined by a spectrophotometer (NanoDrop 2000c, USA). After adjusted at an amount of 1000  $\mu$ g, the RNA was reversed to single-strand cDNA according to the protocol of the kit (TaKaRa, Japan). All the sequences of primers were queried using the NCBI BLAST database for homology comparison ( $\beta$ -actin, forward 5'-CAGGAAGGGATGGAAGGTCTC-3' and reverse 5'-TACCACCCACTTGGCAGACC-3'; E-cadherin, forward 5'-AGGCCAAGCAGCAGTACATT-3' and reverse 5'-ATTCCATCCAGCACATCCA-3'; N-cadherin, forward 5'-AGGTTTGCCAGTGTGACTCC-3' and reverse 5'-TGATGATGCAGAGCAGGATG-3'; Vimentin, forward 5'-ATCCAAGTTTGCTGACCTCTCTGA-3' and reverse 5'-GACTGCACCTGTCTCCGGTACTC-3'). The PCR assays were performed using the RG-3000 Rotor-Gene 4 channel Multiplexing System (ABI Stepone, USA). Gene expressions were analyzed as relative RNA levels with  $\beta$ -actin as an internal control.

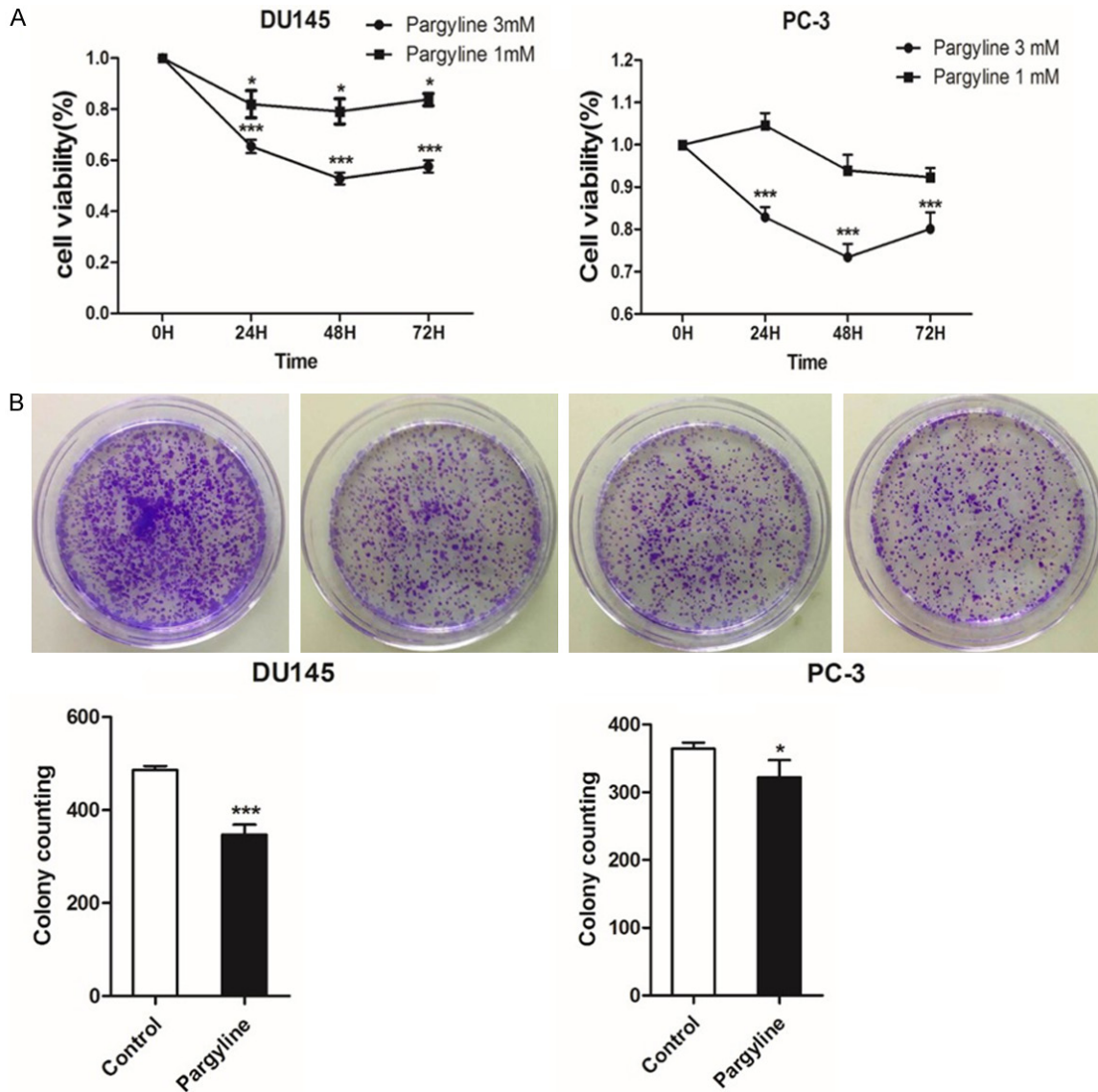
### *Online data analysis*

Prostate cancer patients' gene data and clinical data (GSE46177) was obtained from Gene Expression Omnibus (GEO) site. All cases were divided into two groups based on the methylation level (top 25%: high vs bottom 75%: low), and Kaplan-Meier analysis and conducted. We also searched the protein network in STRING to suggest the relationships based on published papers.

### *Statistical analysis*

Data are expressed as the mean  $\pm$  SEM. Differences were evaluated by student's test using SPSS 19.0 software. A value of  $P < 0.05$  was considered statistically significant.

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**Figure 1.** The effect of different dose of pargyline on cell viability and proliferation in DU145 and PC-3. A. With the treatment of pargyline at 0, 1, 3 mM for different time (0-72 H). Pargyline at 3 mM inhibited the cell viability in both cell lines ( $***P<0.001$ ). Treatment with low concentration at 1 mM decreased cell viability rate of DU145 cells ( $*P<0.05$ ) while had no significant effect on PC-3 cells ( $P>0.05$ ). B. Treatment with 3 mM pargyline significantly decreased the numbers of colony formation in both cell lines ( $*P<0.05$ ,  $***P<0.001$ ).

### Results

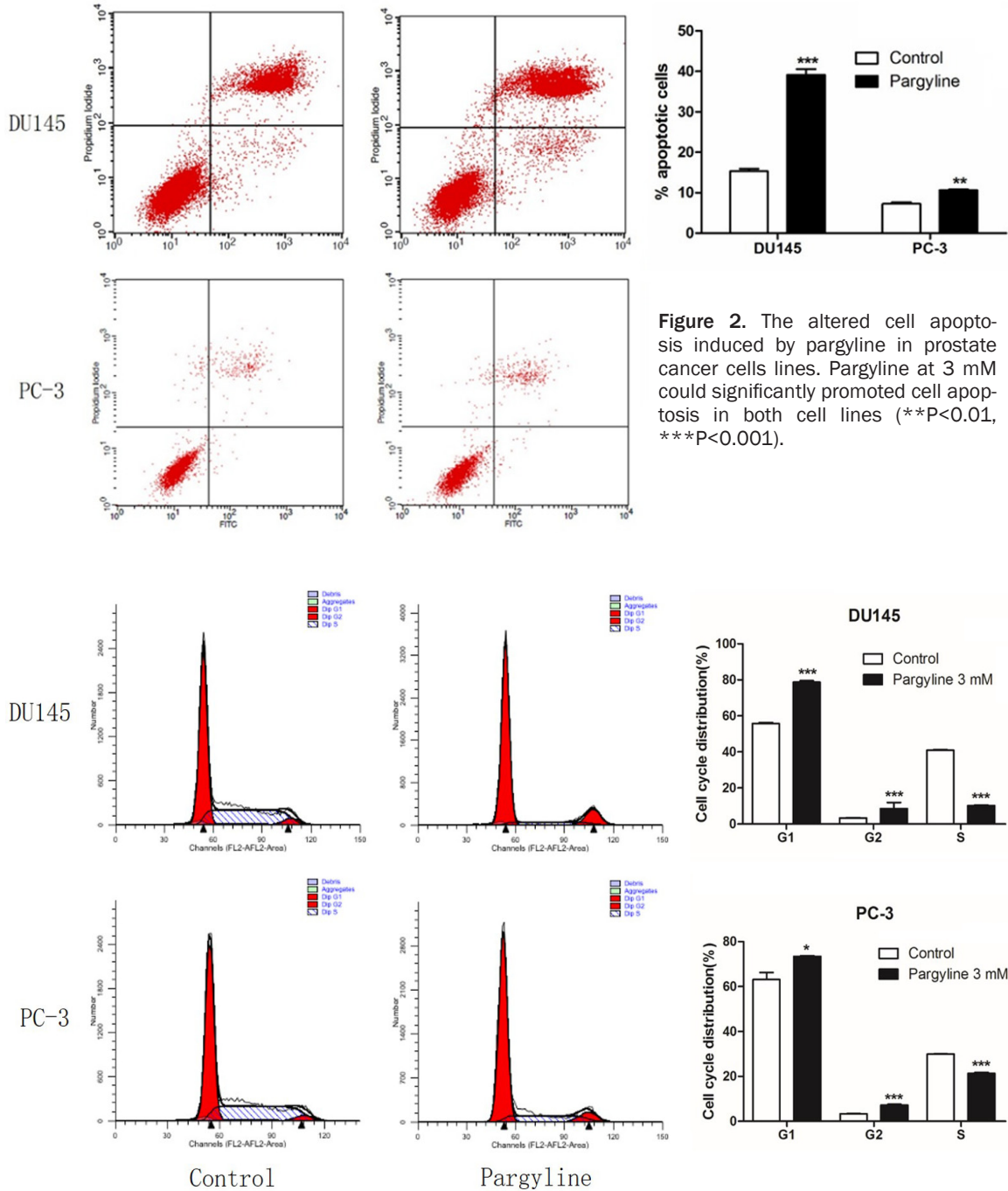
#### *Pargyline reduced cellular proliferation of human prostate cancer DU145 and PC-3 cell lines*

LSD1 had been shown to be overexpressed in some prostate cancers. According to the results of our preliminary experimental basis [12], we had shown LSD1 was overexpressed in prostate cancer tissue and pargyline was an effective inhibitor of LSD1 for androgen-dependent prostate cancer model [19]. However, the ef-

fects of pargyline on androgen-independent prostate cell lines remain unclear. In order to explore the effects of LSD1 inhibitor on the proliferation of DU145 and PC-3 cells, we used Cell Counting Kit-8 (CCK8) to determine the cell viability at different concentrations (0 mM, 1 mM, 3 mM) of pargyline for different time. CCK8 assay indicated that pargyline at a high concentration of 3 mM markedly decreased the proliferation rate in both cell lines (**Figure 1A**,  $***P<0.001$ ). Treatment of low concentration at 1 mM decreased cell proliferation of DU145 cells ( $*P<0.05$ ) while had



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**Figure 2.** The altered cell apoptosis induced by pargyline in prostate cancer cells lines. Pargyline at 3 mM could significantly promoted cell apoptosis in both cell lines (\*\*P<0.01, \*\*\*P<0.001).

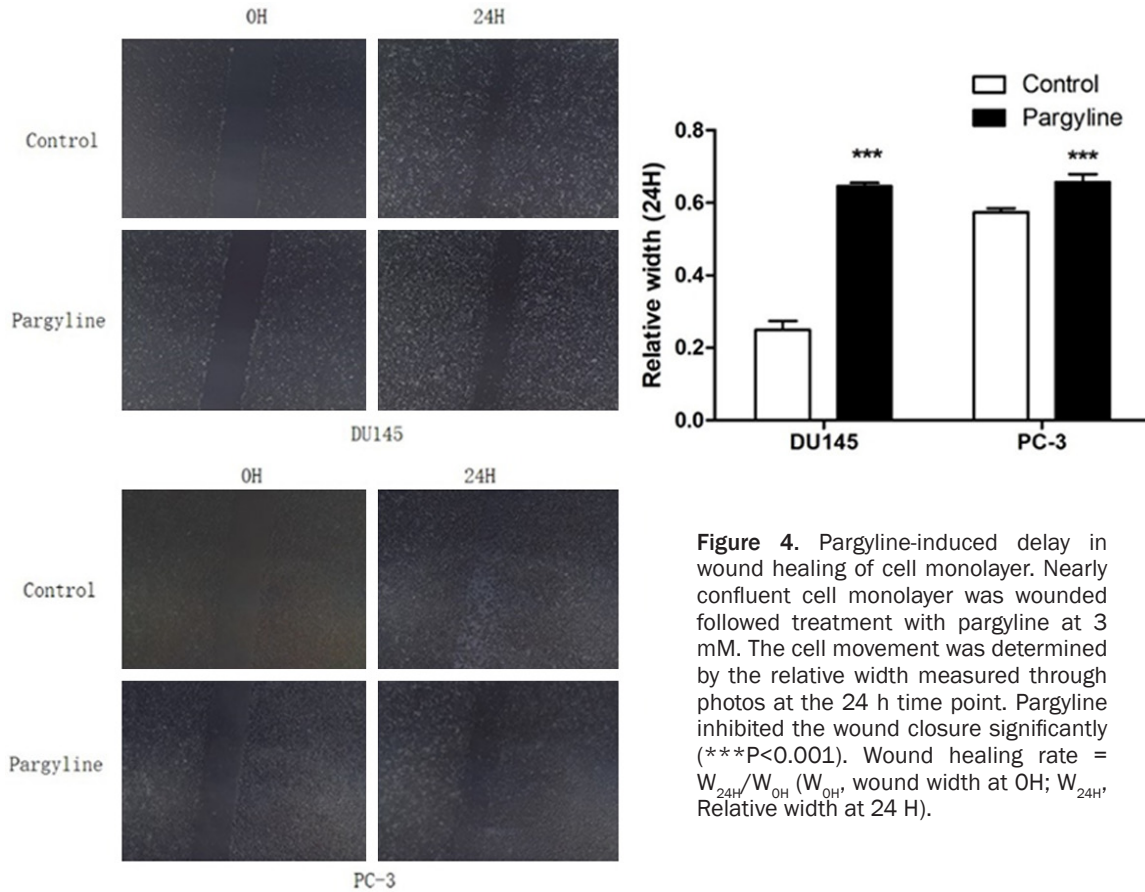
**Figure 3.** Pargyline-induced cell cycle arrest. Changes in the distribution of cell cycle showed the S phrase was significantly inhibited, resulting in increased cells arrested in G1 and G2 phrases (\*\*P<0.001).

no significant effect on PC-3 cells ( $P>0.05$ ). Treatment with 3 mM pargyline significantly decreased the numbers of colony formation in both cell lines (Figure 1B,  $*P<0.05$ ,  $***P<0.001$ ). Meanwhile, these experiments were carried out for determining the optimal condition for concentration and incubation time of pargyline (3 mM for 48 H).

### *Pargyline promoted cellular apoptosis of DU145 and PC-3 cell lines*

The observation described above suggested pargyline could significantly decrease the proliferation ability of prostate cancer cells. To explore the effect of pargyline on DU145 and PC-3 cell apoptosis, we used the flow cytomet-

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**Figure 4.** Pargyline-induced delay in wound healing of cell monolayer. Nearly confluent cell monolayer was wounded followed treatment with pargyline at 3 mM. The cell movement was determined by the relative width measured through photos at the 24 h time point. Pargyline inhibited the wound closure significantly (\*\*\*) ( $P < 0.001$ ). Wound healing rate =  $W_{24H}/W_{0H}$  ( $W_{0H}$ , wound width at 0H;  $W_{24H}$ , Relative width at 24 H).

ric analysis with cells stained with PI/Annexin V. The results indicated pargyline could significantly promote cell apoptotic rate in both cell lines compared with control respectively (**Figure 2**, \*\*\* $P < 0.001$ , \*\* $P < 0.01$ ).

### *Pargyline induced cell-cycle arrest in DU145 and PC-3 cell lines*

To explore the mechanism of pargyline to inhibit proliferation of Du145 and PC-3 cells and to investigate whether pargyline was able to affect DNA synthesis, we analyzed cell cycle progression using a flow cytometer. The alternation on cell-cycle progression induced by pargyline showed the fraction of  $G_1$  and  $G_2$  phase cells significantly increased. Meanwhile, the proportion of cells in S phase decreased accordingly (**Figure 3**, \* $P < 0.05$ , \*\*\* $P < 0.001$ ).

### *Pargyline limited cell motility/migration of DU145 and PC-3 cells*

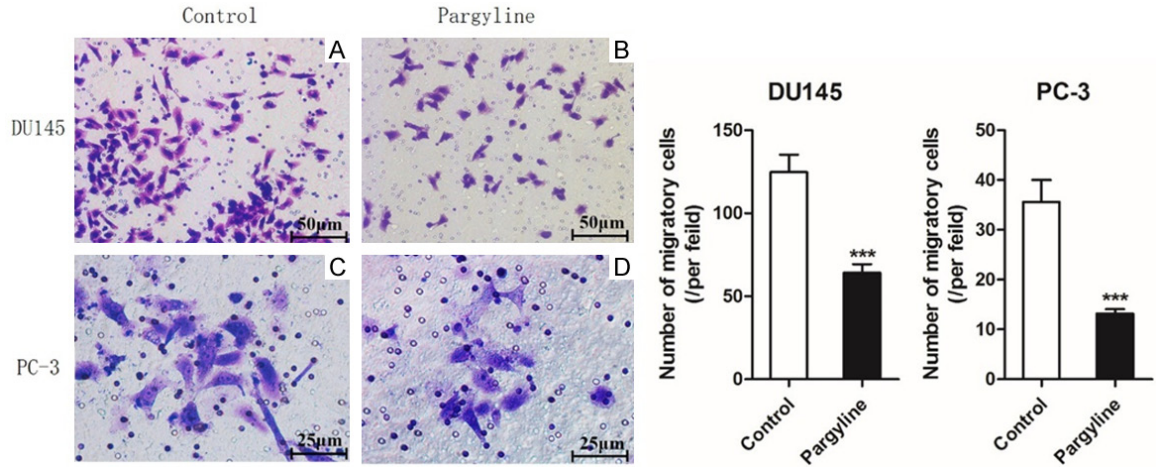
Our previous study of prostate cancer showed overexpressed LSD1 related to the developed

clinical features [12]. To explore the effect of pargyline on cell motility, wound healing assay were used to examine the ability of cell movement. **Figure 4** showed that both cell lines with a treatment of pargyline (3 mM) had wider scratch than the control significantly (\*\*\*) ( $P < 0.001$ ). In accordance with the delayed wound healing, the cells migrating through the micro-pore significantly decreased after treated with pargyline (**Figure 5**).

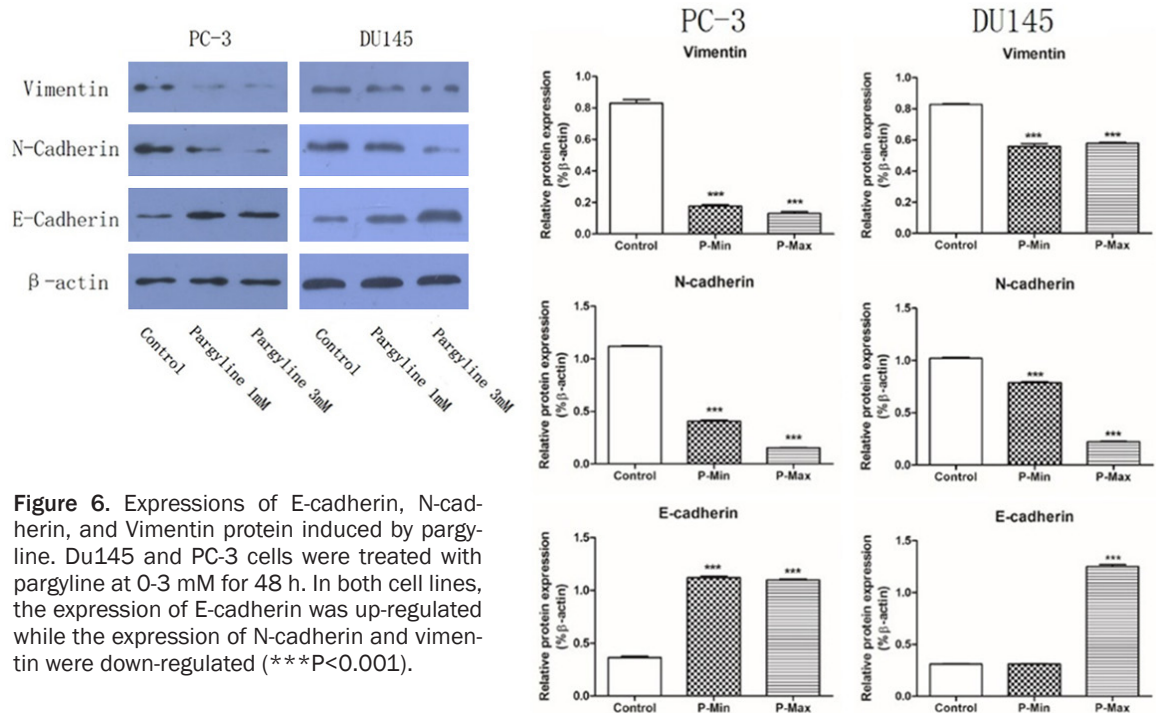
### *Pargyline inhibited the EMT process in DU145 and PC-3 cells*

To explore the possible effects on EMT process induced by pargyline, western blot and RT-qPCR were used to determine the relative protein and gene expression of core genes in EMT respectively. Core proteins in EMT indicated significant differences compared to the control (**Figure 6**). Expressions of vimentin and N-cadherin significantly decreased in both cell lines under treatment with pargyline for 48 hours. While expression of E-cadherin increased significantly. Uniformly, **Figure 7** demon-

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**Figure 5.** The effect of pargyline on cells migrating through transwell chamber. The cells were counted after 24 h treated with pargyline at 3 mM. Pargyline could block the cell migration in both cell lines (\*\*\*) $P < 0.001$ .



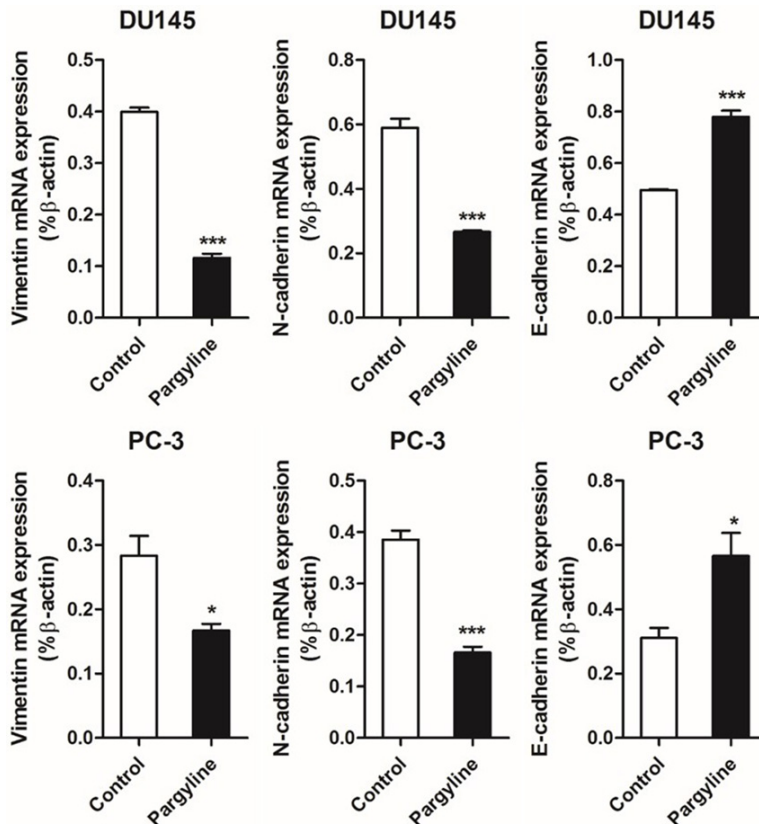
**Figure 6.** Expressions of E-cadherin, N-cadherin, and Vimentin protein induced by pargyline. Du145 and PC-3 cells were treated with pargyline at 0-3 mM for 48 h. In both cell lines, the expression of E-cadherin was up-regulated while the expression of N-cadherin and vimentin were down-regulated (\*\*\*) $P < 0.001$ .

strated increased E-cadherin, decreased Vimentin and N-cadherin in both cell lines treated with pargyline (3 mM) for 48 hours.

### Discussion

LSD1 had been considered to act as a tumor promoter in different kinds of carcinoma, involving a number of processes from adipogenesis to cell-cell adhesion to viral latency, regulating several cellular pathways related with proliferation, development, and cell cycle con-

trol [20, 21]. LSD1 was overexpressed in many solid tumors including prostate cancer and was regarded as a potentially important target for therapeutic drugs [22]. Numerous chemical or gene inhibitors of LSD1 had been researched in recent years and some of them have entered the clinical arena [23]. However the regulation mechanisms remained largely unknown. In our prior study, we suggested higher expression of LSD1 accompanied with lower expression of E-cadherin correlated with higher Gleason Scores, higher PSA, and more



**Figure 7.** mRNA expressions of E-cadherin, N-cadherin, and Vimentin induced by pargyline. Du145 and PC-3 cells were treated with pargyline at 0, 3 mM for 48 h. RT-qPCR was used to exam the gene expressions of EMT. The expression of E-cadherin was up-regulated while the expression of N-cadherin and vimentin were down-regulated (\* $P < 0.05$ , \*\*\* $P < 0.001$ ).

advanced clinical stage [12]. We supposed that inhibition of LSD1 by pargyline could have an effect of tumor suppression on prostate cancer.

Firstly, a hallmark of cancer cell was sustaining proliferative signaling, accompanying enhancing DNA replication. LSD1 acted as an important role in control of chromosome segregation, cell cycle arrest and apoptosis [24]. LSD1 was up-regulated in most of solid tumors and acted as tumor promotor. LSD1 worked as co-activator or co-repressor in the form of LSD1-containing complexes with CoREST, Snail and other functional proteins [24, 25]. In addition, LSD1 also co-localized with some nuclear receptors including AR, mediating the transcription of downstream gene expressions [26]. LSD1 was a well-defined target of MAOIs including pargyline which was already used therapeutically. Therefore researches of LSD1 inhibitors might offer proofs for a novel thera-

peutic target for PCa and enhance the importance of developing additional LSD1 inhibitors. In our study, we demonstrated that pargyline had effective tumor repression on androgen-independent prostate cancer cell lines. Pargyline significantly inhibited the cellular proliferation through increased apoptosis and arrest in cell cycle.

Secondly, capability of motility and transition was another important feature of cancer cells. In vitro, we demonstrated the effects of LSD1 inhibitor on cellular motility and EMT process. 3 mM pargyline significantly delayed the wound healing and prevented the cancer cells from migrating through the micropores. As mentioned before, LSD1 was a well-defined target of MAOIs. Snail genes were crucial to the function of the promotor of E-cadherin. The AO domain of LSD1 and the SNAG domain of Snail were important for their association, and this interaction was regulat-

ed by CoREST [24]. However, the related networking and regulation network still remained largely unknown. All of these components were coordinated by histone modifications, specifically methylation and acetylation of lysine 4 on histone H3 (H3K4), H3K9 and H3K27 [10]. H3K27 methylation and H3K9 methylation were marked as repressive transcription, while H3K4 methylation and H3K9 acetylation were marked as active transcription. Most of the researches were investigated in breast cancer and lung cancer. In prostate cancer, pargyline was found to block demethylation of H3K9 marks and to inhibit androgen mediated AR dependent gene activation [27]. Dong et al had showed that methylation of H3K9 was critical for promoter DNA methylation of E-cadherin in TGF- $\beta$ -induced EMT model in breast cancer cell lines [28]. We supplied LSD1 inhibitor pargyline also repressed EMT process in androgen-independent PCa cell lines. Additionally, there were several non-histone client proteins



of importance. LSD1 mediated demethylation of the tumor suppressor p53 by inhibiting its function [29]. However, demethylation of the DNA methyltransferase (DNMT1) was necessary for maintaining DNA methylation activity [30].

LSD1 participated in the histone and non-histone dynamic methylation process. It was interesting to identify the mechanism how to regulate the EMT process by LSD1. We used the online analyzing data on STRING, showing LSD1 probably had relationships with EMT genes through some pathways (Figure S1). In addition to histone demethylation regulation, the direct demethylations contributed importantly to gene expression regulation. It would be interesting to determine whether LSD1 had non-histone regulation on EMT in PCa. As mentioned before, LSD1 could modulate other epigenetic regulatory proteins related to DNA methylation. We found the DNA methylation of E-cadherin was significantly changed in PCa by analyzing the clinical data from GEO (GSE46177). The Kaplan-Meier plot demonstrated that lower E-cadherin methylation was associated with shorter disease-specific survival of prostate cancer (Figure S2). More studies were required to explore if LSD1 had a direct effects on E-cadherin in further studies.

In summary, our present study demonstrated that LSD1 inhibitor pargyline could inhibit cellular proliferation, motility and EMT process in androgen-independent prostate cancer cell lines. LSD1 inhibitor might offer an attractive therapeutic target for prostate cancer in different stages.

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#### Disclosure of conflict of interest

None.

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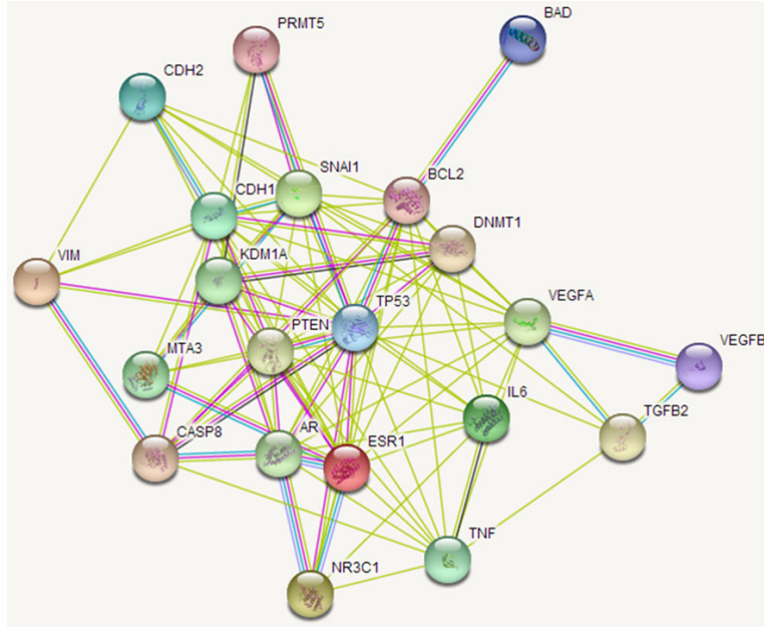
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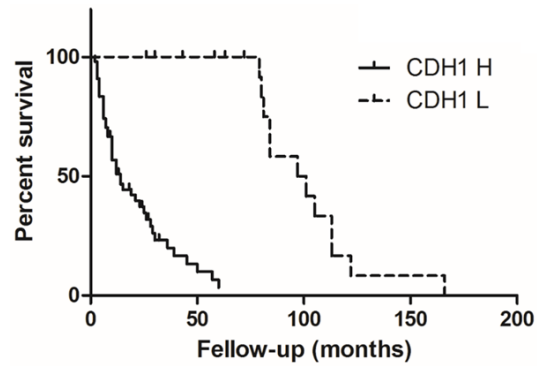
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## LSD1 inhibitor repressed PCa in vitro



**Figure S1.** Protein-protein interactions. Predicted protein-protein interactions generated following analysis of different peptides input into the STRING database. LSD1 probably had relationships with EMT genes through some pathways.



**Figure S2.** Lower E-cadherin methylation was associated with shorter disease-specific survival of prostate cancer.