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Original Article

Lycorine inhibits tumor growth of human osteosarcoma cells by blocking Wnt/β-catenin, ERK1/2/MAPK and PI3K/AKT signaling pathway

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Abstract: Osteosarcoma (OS) is the most common type of primary bone cancer. Even with advances in early diagnosis and aggressive treatment, the overall prognosis for OS remains to be further elevated. Lycorine was an isoquinoline alkaloid mainly existed in the bulb of lyco salvia miltiorrhiza and was shown to inhibit several types of cancer. In the present study, we investigated the anti-OS activity of lycorine and the possible underlying mechanism. We found that lycorine inhibited cell proliferation of human OS cells while had lower cytotoxcity against normal cells, and triggered cell cycle arrest at the G1/S transition. Moreover, we validated that lycorine promoted apoptosis via death receptor pathway and mitochondrial pathway, suppressed migration and invasion by reversing epithelial mesenchymal transition (EMT) and suppressing the degradation of extracellular matrix (ECM) *in vitro*. In addition, orthotopic implantation model of 143B OS cells further confirmed that lycorine suppressed OS growth and lung metastasis *in vivo*. Mechanically, lycorine reduced the protein level of β-catenin and its' downstream molecule c-Myc. Furthermore, lycorine also decreased the phosphorylation of ERK1/2 and AKT. Together, our results reveal that lycorine may inhibit tumor growth of OS cells possibly through suppressing Wnt/β-catenin, ERK1/2 and PI3K/AKT signaling pathway.

Keywords: Lycorine, osteosarcoma, Wnt/β-catenin, ERK1/2, PI3K/AKT

Introduction

Osteosarcoma (OS) is a malignant bone tumor originating from mesenchymal cells with osteogenic potential. OS accounts for 50% of all bone tumors and is the most common primary malignancies in children and adolescent [1]. Although long-term disease-free survival rate of OS patients reaches 60-75% [2], OS is particularly prone to distant lung metastases, and when lung metastases is diagnosed, the survival rate drops sharply to 30% [3, 4]. Current treatment strategies are limb salvage surgery and neoadjuvant chemotherapy. The common-used clinical chemotherapy agents for OS treatment include methotrexate (MTX), adriamycin (ADM) and ifosfamide (IFO). Although these drugs have some effects, most of them are usually easy to develop drug resistance and have strong cytotoxicity against normal cells which may cause bone marrow suppression, kidney toxicity and liver dysfunction [5]. Therefore, it is urgent to explore more effective and promising drugs for the treatment of OS.

Growing evidences have indicated that traditional Chinese medicine may be valuable drug resources for the treatment of various cancers with mild adverse effects and less drug resistance. Lycorine is an isoquinoline alkaloid with high content in the bulb of lycoraceae [6]. In addition to its anti-viral, anti-malarial, anti-bacterial and anti-inflammatory activities [7], lycorine also has profound anti-tumor potency against various cancers including liver cancer [8], bladder cancer [9], ovarian cancer [10], kidney cancer [11], leukemia [12], prostate cancer [6] and multiple myeloma [13]. Meanwhile, it has been reported that lycorine was cytotoxic to the adriamycin-resistant leukemia cells where-

as has lower toxicity against normal cells [14], which implies that lycorine may be a potentially effective and safe drug candidate for cancer treatment.

In the current study, we investigated the anticancer effect of lycorine on human OS cells and probed the possible underlying mechanisms. We found that lycorine validly inhibited the survival of human OS cells while had lower toxicity against normal cells. Concurrently, lycorine induced the cell cycle arrest at the G1/S transition, promoted OS cells apoptosis via the death receptor pathway and the mitochondrial pathway, inhibited the migration and invasion through reversing the epithelial mesenchymal transition (EMT) process and accelerating the degradation of extracellular matrix (ECM) of OS cells *in vitro*. Orthotopic tumor model further confirmed that lycorine significantly inhibited orthotopic OS tumor growth and lung metastasis *in vivo*. Molecular mechanism analysis revealed that lycorine inhibited the activation of classical Wnt/β-catenin, ERK1/2/MAPK and PI3K/AKT signaling pathway. Collectively, our results intensively suggest that lycorine may be a potentially effective drug for the treatment of OS.

Materials and methods

Reagents

Lycorine (purity ≥98%; Ruifensi, Chengdu, China) was dissolved into 20 mM with dimethyl sulfoxide (DMSO; Sigma, California, USA), and stored at -80°C. Luciferase reporter plasmid p-BGluc-TCF/LEF, p-BGluc-ELk1/SRF, p-BGluc-RBP/JK, p-BGluc-NFAT, p-BGluc-E2F/DP1 were provided by Dr. Tongchuan He (University of Chicago Medical Center, USA). Rabbit or Mouse SP detection kit and sodium citrate were purchased from Zhongshanjinqiao (Beijing, China). Mouse monoclonal antibody against β-actin, PCNA (Proliferating cell nuclear antigen), Bcl-2 (B cell lymphoma/leukemia-2), Snail, E-cadherin, Caspase8, Caspase9, rabbit polyclonal antibody against N-cadherin, Vementin, MMP-2 (Matrix metalloproteinase-2), MMP-7 (Matrix metalloproteinase-7) MMP-9 (Matrix metalloproteinase-9), Bad (Bcl-2-associated agonist of cell death), Bax (Bcl-2-associated X protein), Fadd (Fas-associating protein with a novel death domain), Caspase3, Cleaved-Caspase3 (c-Caspase3), PARP (Poly ADP-ribose polymerase), Cleaved-PARP (c-PARP), c-Myc, CyclinD1, CyclinE1, β-catenin, ERK1/2 (Extracellular regulated protein kinases), p-ERK1/2 (T202/Y204), AKT, p-AKT (S473), p-AKT (T308) were obtained from Cell Signaling Technology (Boston, USA).

Cell culture

Human liver cell MIHA, human proximal renal tubular epithelial cell HK-2, human brain glial cell HEB, human bone marrow stromal cell HS-5, and human OS cell lines 143B, MG63, SaoS2, U2OS were provided by Key Laboratory of Diagnostic Medicine designated by the Chinese Ministry of Education, Chongqing Medical University. All cells were cultured in Dulbecco's modified Eagle medium (DMEM; Hyclone, Utah, USA) supplemented with 10% fetal bovine serum (FBS; ExCell Bio, Shanghai, China), 1% penicillin and streptomycin (100 IU/ml; Hyclone, Utah, USA), and incubated at 37°C in 5% humidified CO_2.

Crystal violet dye assay

In brief, 143B, MG63, SaoS2 and U2OS cells were seeded into 24-well (5×10^4 cells/well) plates (Corning, New York, USA) and treated with various concentrations lycorine (0 μM, 1 μM, 2 μM, 4 μM, 8 μM). At 72 h after lycorine treatment, cells were stained with crystal violet (Beyotime, Shanghai, China) to visualize the cell viability. All experiments were performed in triplicate.

MTT assay

In brief, MIHA, HK-2, HEB, HS-5,143B, MG63, SaoS2 and U2OS cells were seeded into 96-well plates (3×10^3 cells/well) and treated with various concentrations of lycorine for 48 h. Then, cells were incubated with 5 mg/ml MTT solution (Sigma, California, USA) for 4 h. MTT solution was discarded and DMSO (150 μL/well) was added to fully dissolve the MTT formazan. The cell plates were then placed in a multifunctional enzyme labeling instrument (BioTek, Hongkong, China) to detect the absorbance at 492 nm. All experiments were performed in triplicate.

Colony formation assay

In brief, 143B and MG63 cells were seeded into 6-well plates (150 cells/well) and allowed to grow for 24 h, cells were then treated with

various concentrations of lycorine for 7 days, respectively. The colonies were fixed with 4% paraformaldehyde for 10 min, stained with 0.1% crystal violet for 5 min, and the colony formation rate was calculated: colony formation rate (%) = number of colony formation/number of inoculated cells ×100%. All experiments were performed in triplicate.

Wound-healing migration assay

Briefly, 143B and MG63 cells were allowed to grow into full confluence in 6-well plates, then the cells wounds were created by scratching cells with a 10 μL pipette. Cells were washed with PBS gently for 3 times, and then incubated with fresh DMEM containing different concentrations of lycorine. Three different fields of each wound were randomly photographed with inverted fluorescence microscope (×100; Nikon, Japan) after incubating for 18 h and 24 h. Wound-healing migration rate (%) = (0 h scratch width - end time scratch width)/0 h scratch width ×100%. All experiments were performed in triplicate.

Transwell invasion assay

The invasion ability of OS cells were assessed by transwell chamber assay, as described previously [15]. For cell invasion assay, 143B and MG63 cells were suspended in serum-free medium containing different concentrations of lycorine, and seeded at 5×10^4/well in the upper chamber of matrigel-coated 24-well chambers. After 24 h, non-invaded cells on the upper chamber were removed, and the invaded cells (on the underside of the chamber) were fixed with 4% paraformaldehyde, then stained with crystal violet solution, and photographed under light microscopy (×100). All experiments were performed in triplicate.

Hoechst33258 assay

143B and MG63 cells were allowed to grow into 50~60% confluence in 24-well plates, then treated with different concentrations. At 24 h after lycorine treatment, cells were fixed with 4% paraformaldehyde and stained with 10 ng/ml Hoechst33258 (Solarbio, Beijing, China) for 10 min. The apoptotic cells in three random fields were photographed under light microscopy (×100). All experiments were performed in triplicate.

Flow cytometry

143B and MG63 cells were plated into 60 mm dish. After the cell confluence reached 50~60%, cells were treated with different concentrations of lycorine for 40 h. Then the cells were washed with PBS for 3 times, and re-suspended with 500 μL of PBS, stained with Annexin V-FITC/PI kit (Solarbio, Beijing, China) according to the manufacturer's guidelines to determine cell apoptosis. For cell cycle, cells were fixed with 70% ethanol overnight, stained with PI and subjected to flow cytometry analysis as described previously [16]. All experiments were performed in triplicate.

Luciferase reporter assay

143B cells were transfected with the luciferase reporter plasmids p-BGluc-TCF/LEF, p-BGluc-Elk1/SRF, p-BGluc-RBP/JK, p-BGluc-NFAT, p-BGluc-E2F/DP1 using 15 μL lipofectamine 2000 (Thermo Fisher, Massachusetts, USA) when the cell confluence reached 50~60% in T25 flasks (Corning, New York, USA). At 24 h post transfection, cells were seeded into 24-well plates (5×10^4 cells/well) and treated with different concentrations of lycorine for 12 h. Luciferase activities in cell supernatant were measured using Pierce Gaussia Luciferase Flash Assay Kit (Thermo Fisher, Massachusetts, USA), according to the manufacturer's protocol. All experiments were performed in triplicate.

Western blot

Western blot were performed as previously described [11, 15]. Briefly, 143B cells were treated with various concentrations of lycorine for 48 h, then washed with PBS for 3 times and harvested by centrifugation at 4800 g for 5 min at 4°C. Cells were re-suspended in Western and IP Cell lysis buffer (Boster, Wuhan, China) supplemented with 1% protease and phosphates inhibitors (Roche, Basel, Switzerland). The cell lysates were then centrifuged at 13,000 g for 15 min at 4°C. Subsequently, supernatants were collected in 5× SDS-PAGE sample loading buffer (Boster, Wuhan, China), boiled for 10 min. Protein concentration was determined using a enhanced BCA protein assay kit. Protein samples (40 μg) were separated on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (PVDF;

Millipore, USA). The membranes were incubated overnight with specific primary antibodies (1:1000 dilution) at 4°C after being blocked with 5% Albumin Bovine V (Solarbio, Beijing, China). After washed with 1× TBST, the membranes were incubated with a secondary antibody conjugated with horseradish peroxidase (1:5000 dilution). Protein of interest was visualized by an ECL kit, and photographed by a gel imaging system.

Orthotopic tumor model

BALB/c-nu mice (female, 28 d, 20±2 g; Huafukang, Beijing, China) were cared and handled according to a research protocol approved by the ethics committee of the laboratory animal research of Chongqing Medical University. All mice were bred in a specific pathogen-free (SPF) conditions in a 12 h light-dark cycle at the Department of Animal Center of Chongqing Medical University. In order to allow enough adaptation, the mice were placed in SPF environment for more than 1 week before the experiment. 143B cells (5×10^6 in 70 μL PBS) were inoculated into the proximal tibia of mice. At 7 days post injection, all mice were divided into 5 groups (n = 3) and treated with different doses of lycorine (5 mg/kg, 15 mg/kg and 25 mg/kg) or sodium carboxymethyl cellulose (CMC-Na) by intra-gastric administration every 2 days. The tumor size and body weight were measured every two days for 20 days and then the mice were sacrificed by cervical dislocation to retrieve OS and lung samples.

Hematoxylin-Eosin (H&E) staining

The retrieved OS samples and lung tissues of each group were prepared into paraffin blocks. 5 μm thick sections was prepared from paraffin-embedded OS samples and lung tissues, followed by rehydration using a graded series of 100%, 90%, 80% and 70% ethanol. Sections were then stained by Hematoxylin-Eosin (Solarbio, Beijing, China) according to standard histopathological protocol, and photographed under a light microscope.

Immunohistochemistry (IHC) staining

IHC was carried out as previously reported [11]. Briefly, 0 mg/kg and 15 mg/kg group OS tissues were embedded in paraffin and 5 μm thick sections were prepared. Sections were incubated with primary antibodies. After washing with PBS, sections were incubated with a secondary antibody conjugated with horseradish peroxidase. Protein of interest was visualized by DAB kit, and observed under a light microscope.

Statistical analysis

Results were analyzed by GraphPad Prism 5.0 (San Diego, CA, USA) and SPSS 18.0 (SPSS, Inc., Chicago, IL, USA) software package. Raw data were displayed as mean ± standard deviation (SD), and statistical significance between groups was analyzed by one-way ANOVA. Multiple comparisons between the groups were performed using Tukey's post hoc test. $P<0.05$ is considered statistically significant.

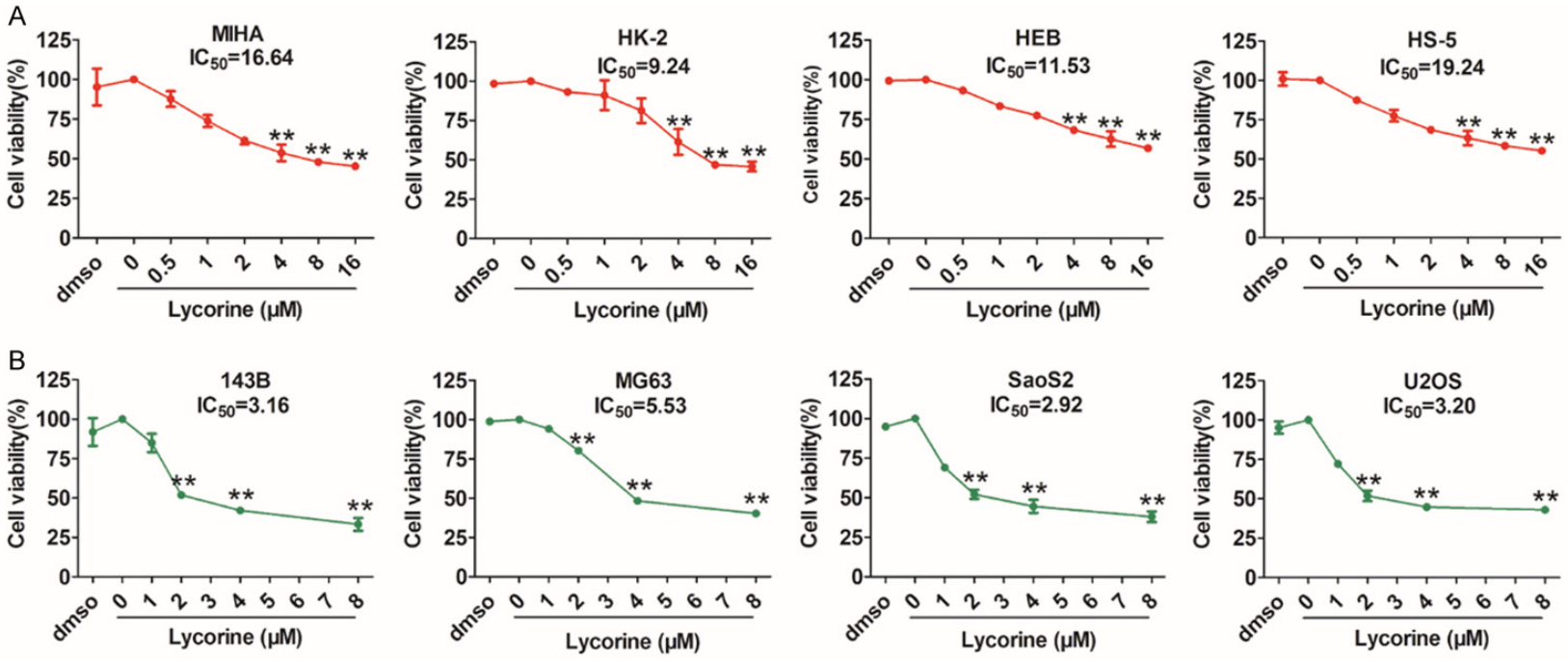
Results

Lycorine inhibits the proliferation ability of OS cells

We firstly thought to determine the effect of lycorine on the proliferation OS cells. By using MTT assay, we found that lycorine effectively inhibited the proliferation of 143B, MG63, SaoS2 and U2OS OS cells with the IC_{50} value of 3.16 μM, 5.53 μM, 2.92 μM, 3.20 μM, respectively (**Figure 1B**). Notably, lycorine exhibited mild inhibitory effect on human normal cells MIHA (human liver cells), HK-2 (human renal proximal tubule cell), HEB (human brain glial cell) and HS-5 (human bone marrow stromal cell) with the IC_{50} of 16.64 μM, 9.24 μM, 11.53 μM, 19.24 μM, respectively (**Figure 1A**). These data imply that lycorine may exhibit profound inhibitory activity against OS cells, while have relative lower toxicity against normal cells. The inhibitory effect of lycorine on OS cells was further confirmed by crystal violet staining assay (**Figure 1C**) and colony formation assay (**Figure 1D** and **1E**). Finally, we validated by western blot that lycorine reduced the protein level of PCNA (**Figure 1F**), which is a well-established proliferation marker. These above results intensively indicate that lycorine may suppress the proliferation of human OS cell and yet may have relative lower toxicity against human normal cells.

Lycorine induces cell cycle arrest at the G1/S phase of OS cells

Next, we decided to determine if the cell cycle of OS cells were disturbed upon lycorine treat-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MIHA
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HK-2
IC50=9.24
HEB
IC50=11.53
HS-5
IC50=19.24
B
143B
IC50=3.16
MG63
IC50=5.53
SaoS2
IC50=2.92
U2OS
IC50=3.20
Cell viability(%)
Lycorine (μM)
dmso
0
0.5
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4
8
16
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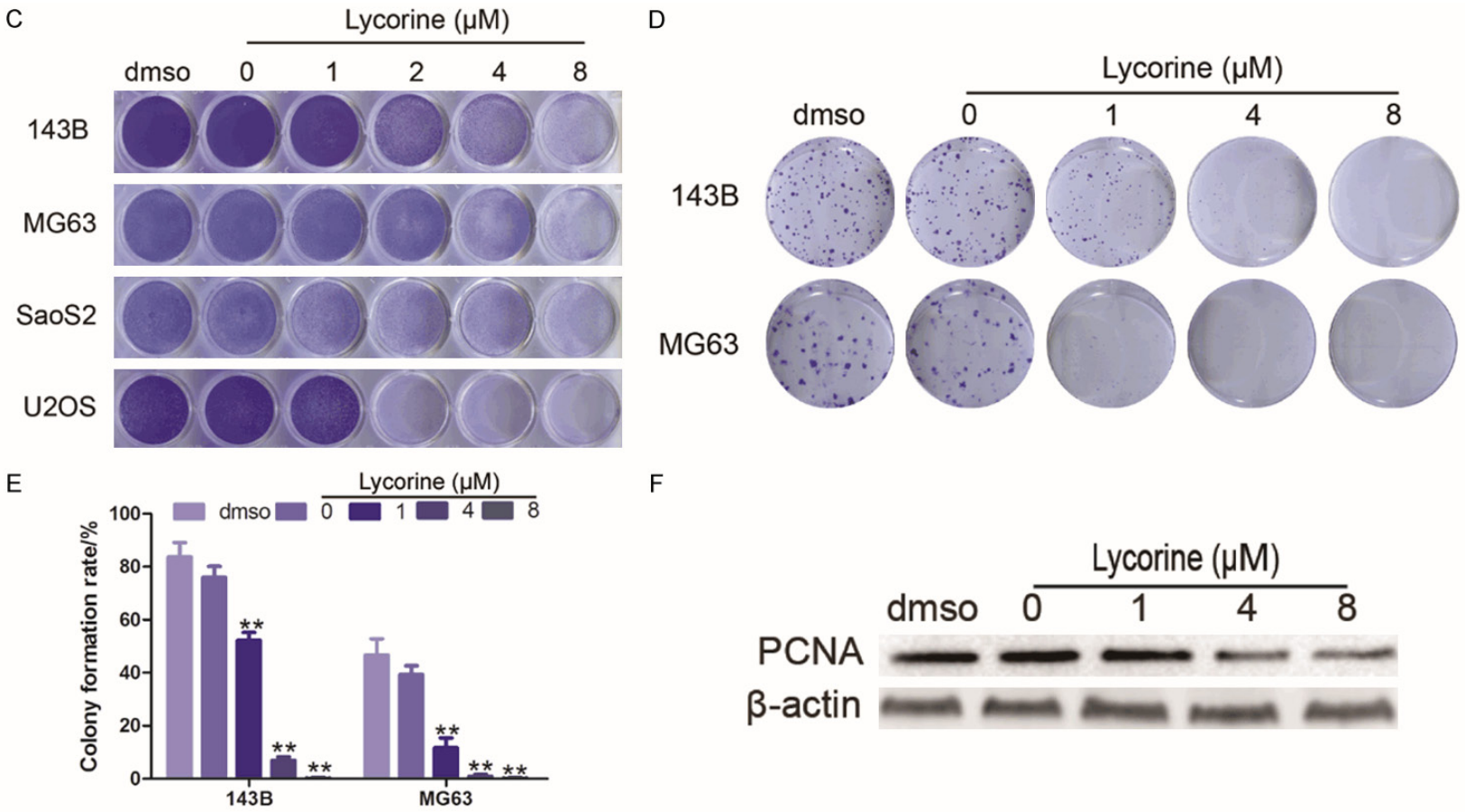


Figure 1. Lycorine inhibits the cells proliferation ability of OS cells. A. Lycorine inhibited OS cells (143B, MG63, SaoS2, U2OS) proliferation (MTT assay). B. Lycorine had relative lower cytotoxcity against normal cells (MIHA, HK-2, HEB, HS-5) (MTT assay). C. Lycorine decreased the proliferative ability of 143B, MG63, SaoS2 and U2OS OS cells (crystal violet staining assay). D, E. Lycorine significantly reduced the colony formation ability of 143B and MG63 OS cells (Colony formation assay). F. Lycorine inhibited the protein level of PCNA in 143B OS cells. PCNA: proliferating cell nuclear antigen. Data are presented as the means ± SD, n = 3, **P*<0.05, ***P*<0.01.

ment. We performed flow cytometry to analyze the cell cycle of 143B OS cells. We found that lycorine increased the percentage of G1 phases whereas decreased the percentage of S and G2/M phase in OS 143B cells (**Figure 2A** and **2B**). Consistent with the flow cytometry results, we validated by western blot that the protein level early G1 phase marker CyclinD1 and late G1 phase marker CyclinE1 was decreased by lycorine treatment (**Figure 2C**). These results suggest that lycorine may trigger the cell cycle arrest at the G1/S phase of 143B OS cells.

Lycorine promotes the apoptosis of OS cells

We then performed Hoechst33258 staining and flow cytometry assay to investigate whether lycorine induces apoptosis of OS cells. As shown in **Figure 3A**, nuclear condensation, fragmentation and chromatin shrinkage of 143B cells treated with lycorine was increased, implying that lycorine may induce apoptosis of OS cells. The results of flow cytometry analysis further validated that the apoptotic proportion of 143B cells was elevated by lycorine treatment (**Figure 3B**). Next, we found that lycorine decreased the level of anti-apoptotic protein Bcl-2 while increased the level of pro-apoptotic proteins Bax and Bad by using western blot (**Figure 3C**). Moreover, the level of cleaved form of Caspase3 (c-Capspase3) which is an important apoptotic marker, and the level of cleaved PARP (c-PARP) which is an indicator of Caspase3 activation were both elevated by lycorine treatment. These findings imply us that lycorine may induce apoptosis of OS cells. Furthermore, we found that lycorine increased the level of death receptor pathway marker Caspase8 and Fadd, as well as the level of the mitochondrial pathway marker Caspase9. These above results intensively suggest that lycorine may promote the apoptosis of OS cells through both mitochondrial pathway and death receptor pathway.

Lycorine suppresses the migration and invasion of OS cells through reversing EMT process and suppressing the degradation of ECM

Next, we employed wound-healing migration assay and transwell chamber assay to determine the effects of lycorine on the migration and invasion of OS cells. We found that lycorine inhibited the migration of 143B and MG63 OS cells, resulting in a decreased wound healing rate (**Figure 4A** and **4B**). Moreover, we demonstrated that lycorine treatment significantly reduced the number of invaded cells which penetrated the Matrigel-coated chamber (**Figure 4C** and **4D**). These results suggest that lycorine may inhibit the migration and invasion of OS cells. EMT and the degradation of ECM are known to play important roles in tumor cell migration and invasion [17]. Therefore, we thought to validate whether EMT process and ECM degradation were affected by lycorine treatment. We found that lycorine decreased the protein level Snail, Vimentin, N-cadherin which are pivotal EMT inducers, whereas increased the protein level of E-cadherin which is an anti-EMT regulator (**Figure 4E**). Moreover, the protein level of ECM degradation markers, MMP-2, MMP-7 and MMP-9 were both downregulated by lycorine as well. Hence, these results suggest that lycorine may inhibit the migration and invasion ability of OS cells possibly by reversing EMT process and suppressing the ECM degradation.

Lycorine blocks Wnt/β-catenin, ERK1/2/MAPK and PI3K/AKT signaling pathway of OS cells

We sought to investigate the molecular basis underlying the anti-OS activity of lycorine. Wnt/β-catenin is an important signal pathway which can regulate the proliferation, migration, invasion and apoptosis of OS cells [17]. We found by luciferase reporter assay that lycorine suppressed reporter activity of p-BGluc-TCF/LEF, which contains TCF/LEF responsive elements and represents β-catenin transcriptional activity (**Figure 5A**). Moreover, we validated that lycorine inhibited the protein level of β-catenin and its' downstream targets c-Myc (**Figure 5B**). These results suggest that lycorine may inhibit Wnt/β-catenin signaling pathway of OS cells.

We also found that lycorine inhibited the reporter activity of p-BGluc-ELK1/SRF, which reflects the activity of ERK1/2 signal pathway (**Figure 5A**). These findings imply us that lycorine may restrain ERK1/2 signal pathway as well. We therefore employed western blot and confirmed that lycorine inhibited ERK1/2 signal pathway, resulting in a decrease of ERK1/2 phosphorylation (**Figure 5B**). Lastly, we explored the effect of lycorine on PI3K/AKT signal pathway as well. As shown in **Figure 5B**, although the protein levels of total AKT were not changed obviously, the phosphorylation of

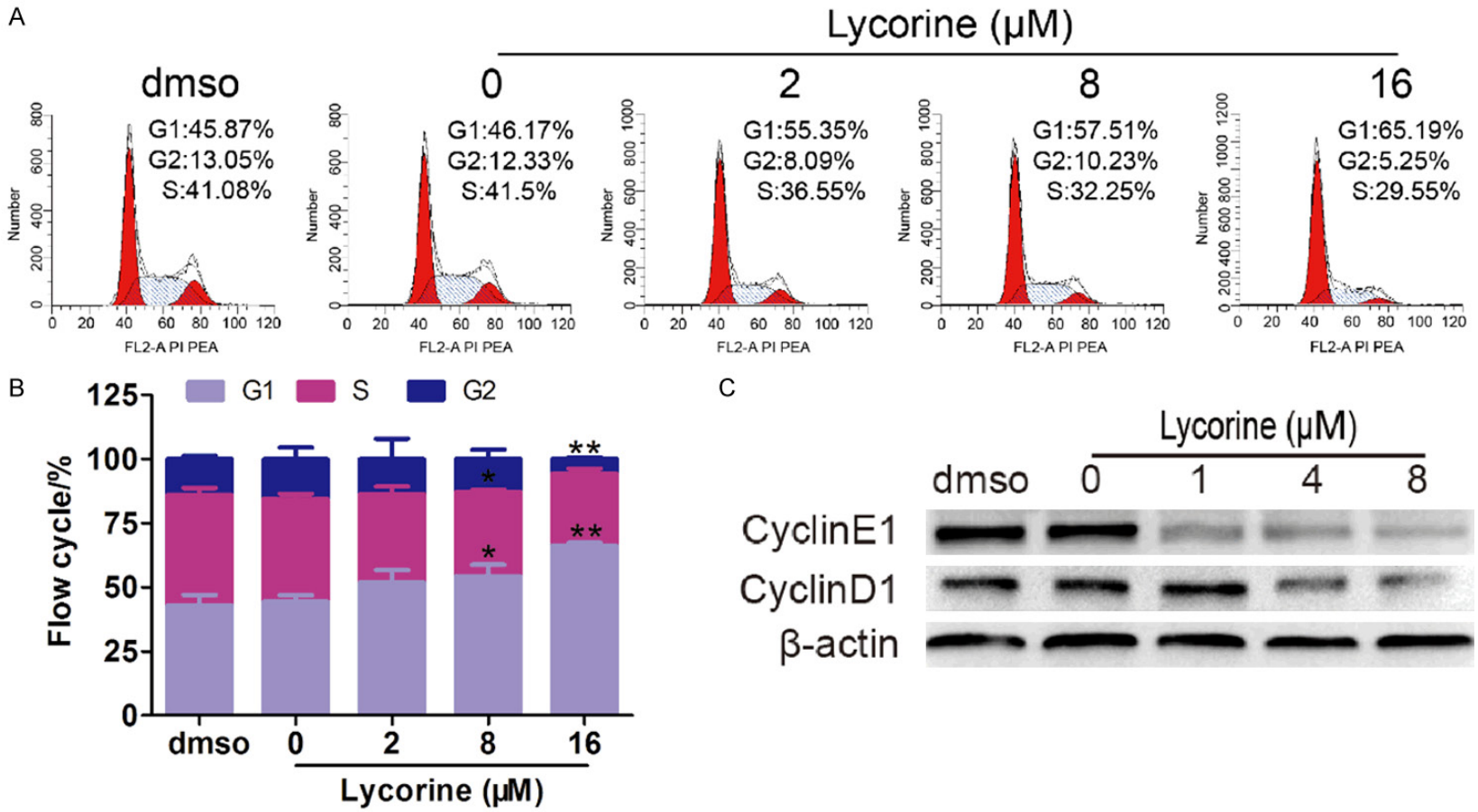
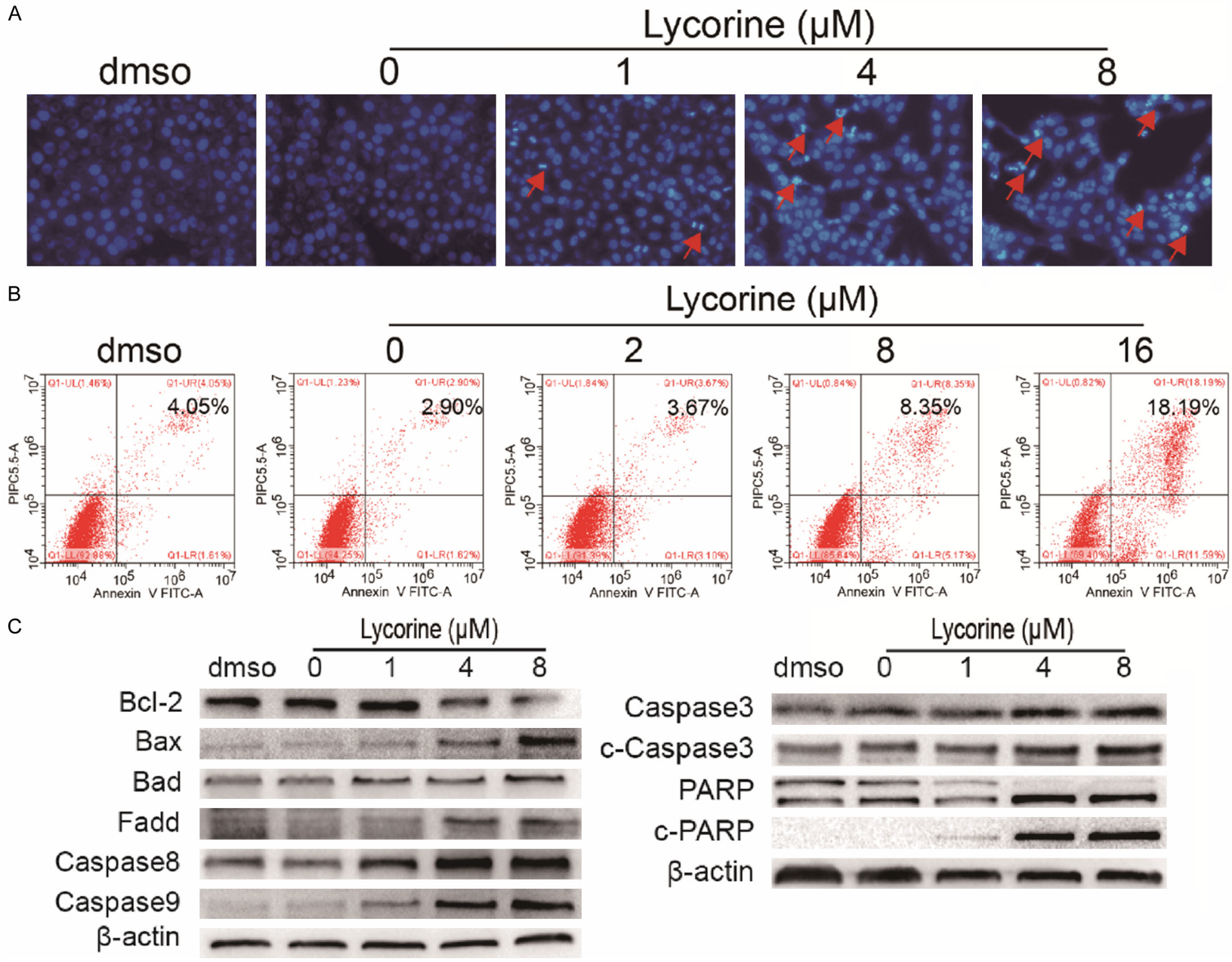


Figure 2. Lycorine induces G1/S cycle arrest of 143B cells. A, B. Lycorine treatment triggered cell cycle arrest at the G1/S transition in 143B OS cells (flow cytometry). C. Lycorine down-regulated the protein expression level of CyclinD1 and CyclinE1 in 143B OS cells (western blot assay). Data are presented as the means ± SD, n = 3, **P*<0.05, ***P*<0.01.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3.67%
8.35%
18.19%
PIPC5.5-A
Annexin V FITC-A
C
Lycorine (μM)
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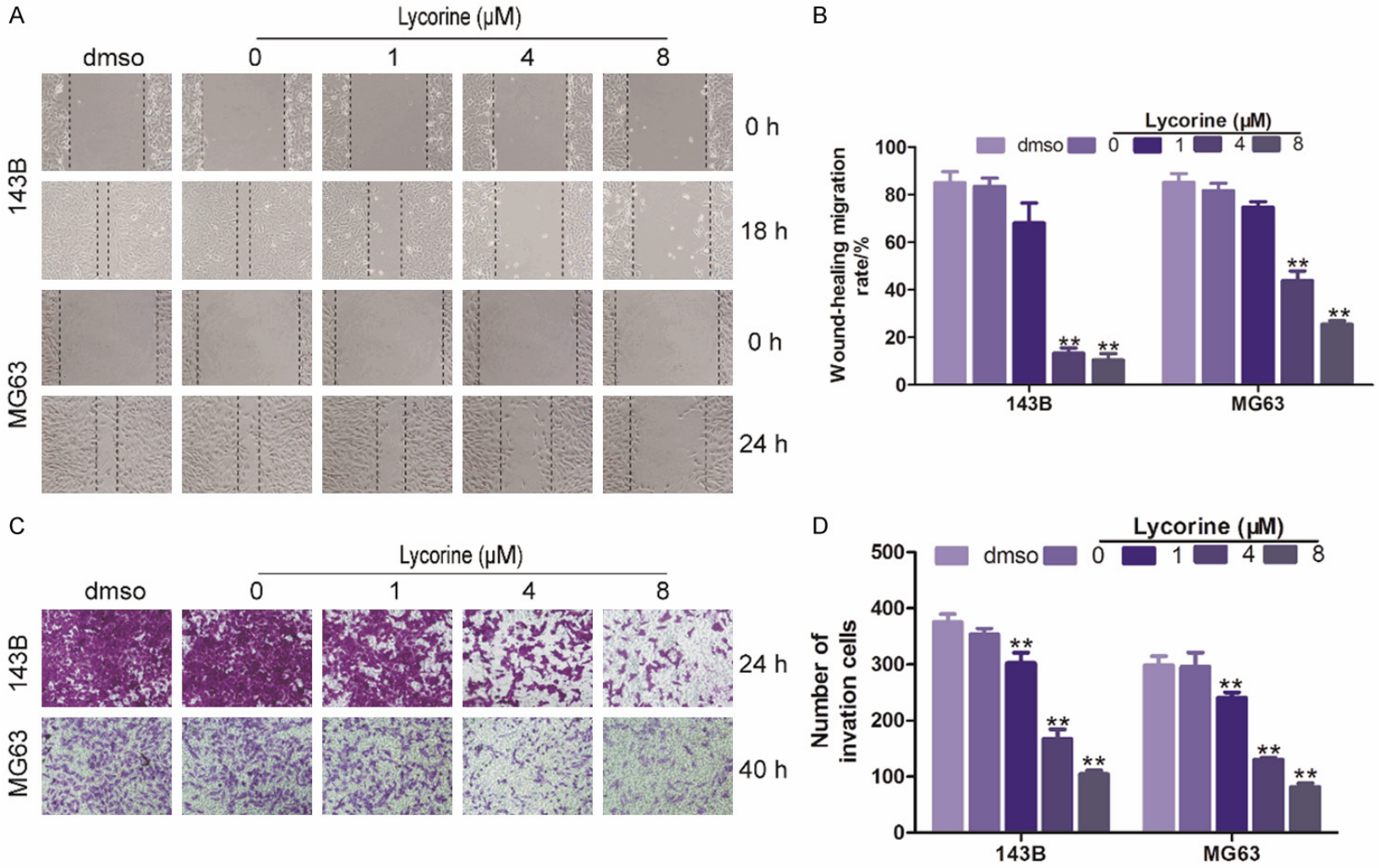
4
8
Bcl-2
Bax
Bad
Fadd
Caspase8
Caspase9
β-actin
Caspase3
c-Caspase3
PARP
c-PARP
β-actin

Figure 3. Lycorine induces the apoptosis of 143B cells. A. Lycorine increased the apoptosis rate of 143B cells (Hoechst33258 assay, ×100). B. Lycorine increased the apoptosis rate of 143B cells (flow cytometry). C. Lycorine promoted the protein level of apoptosis-initiating molecules Bax, Bad, Fadd, Caspase8, Caspase9, decreased the protein level of anti-apoptotic molecule Bcl-2, and increased the protein level of Caspase3, c-Caspase3, PARP and c-PARP in 143B cells. Bcl-2: B cell lymphoma/leukemia-2; Bax: Bcl-2-associated X protein; Bad: Bcl-2-associated agonist of cell death; FADD: Fas-associating protein with a novel death domain; c-Caspase3: Cleaved-Caspase3; PARP: Poly ADP-ribose polymerase; c-PARP: Cleaved-PARP.



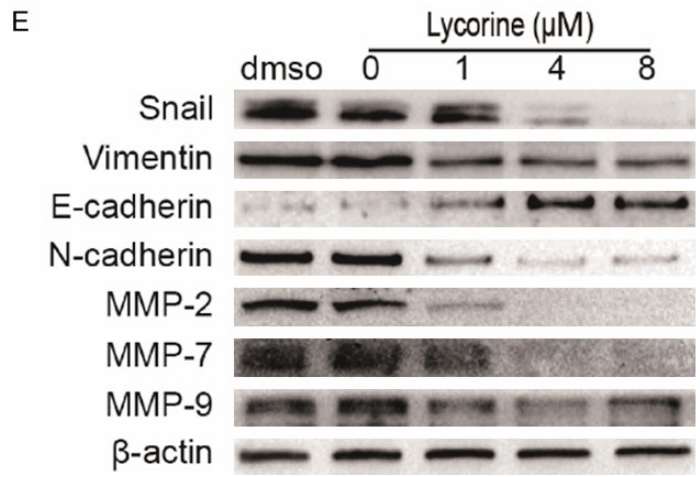


Figure 4. Lycorine suppresses the migration and invasion of 143B and MG63 OS cells. A, B. Lycorine inhibited the migration capacity of 143B and MG63 cells (wound-healing migration, ×100). C, D. Lycorine reduced the invasiveness of 143B and MG63 OS cells (transwell assay, ×100). E. Lycorine increased the protein level of E-cadherin, while inhibited the protein level of Snail, Vimentin, N-cadherin, MMP-2, MMP-7 and MMP-9 (western blot assay). MMP-2: Matrix metalloproteinase-2; MMP-7: Matrix metalloroteinase-7; MMP-9: Matrix metalloproteinase-9. Data are presented as the means ± SD, n = 3, **P*<0.05, ***P*<0.01.

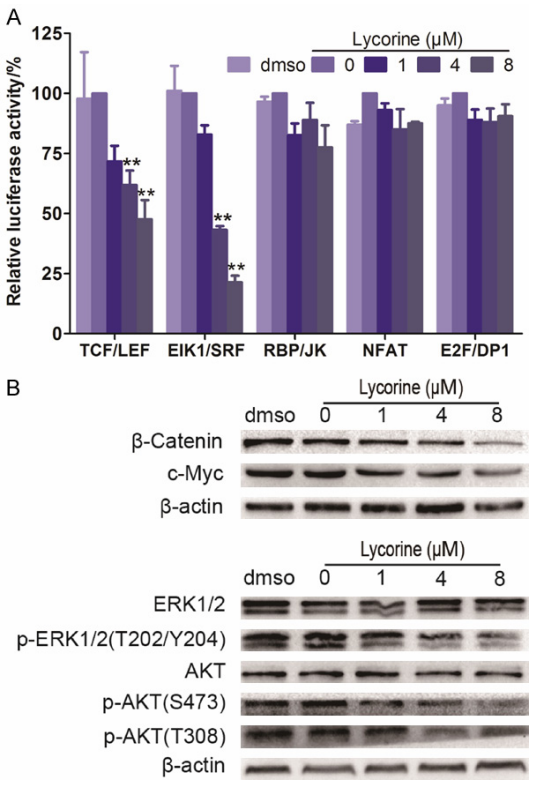


Figure 5. Lycorine inhibits Wnt/β-catenin, ERK1/2/MAPK and PI3K/AKT signaling pathway in 143B OS cells. A. Lycorine inhibited the transcription activity of β-catenin and ERK1/2 in 143B cells (luciferase reporter assay). B. Lycorine decreased the protein level of the related proteins of β-catenin and c-Myc, and reduced the phosphorylation of ERK1/2 and AKT in 143B OS cells by western blot assay. ERK1/2: Extracellular signal-regulated kinase. Data are presented as the means ± SD, n = 3, **P*<0.05, ***P*<0.01.

AKT at Serine 473 (S473) and Threonine 308 (T308) were both decreased by lycorine treatment. The above results suggest that lycorine may inhibit Wnt/β-catenin, ERK1/2 and PI3K/AKT signaling pathway.

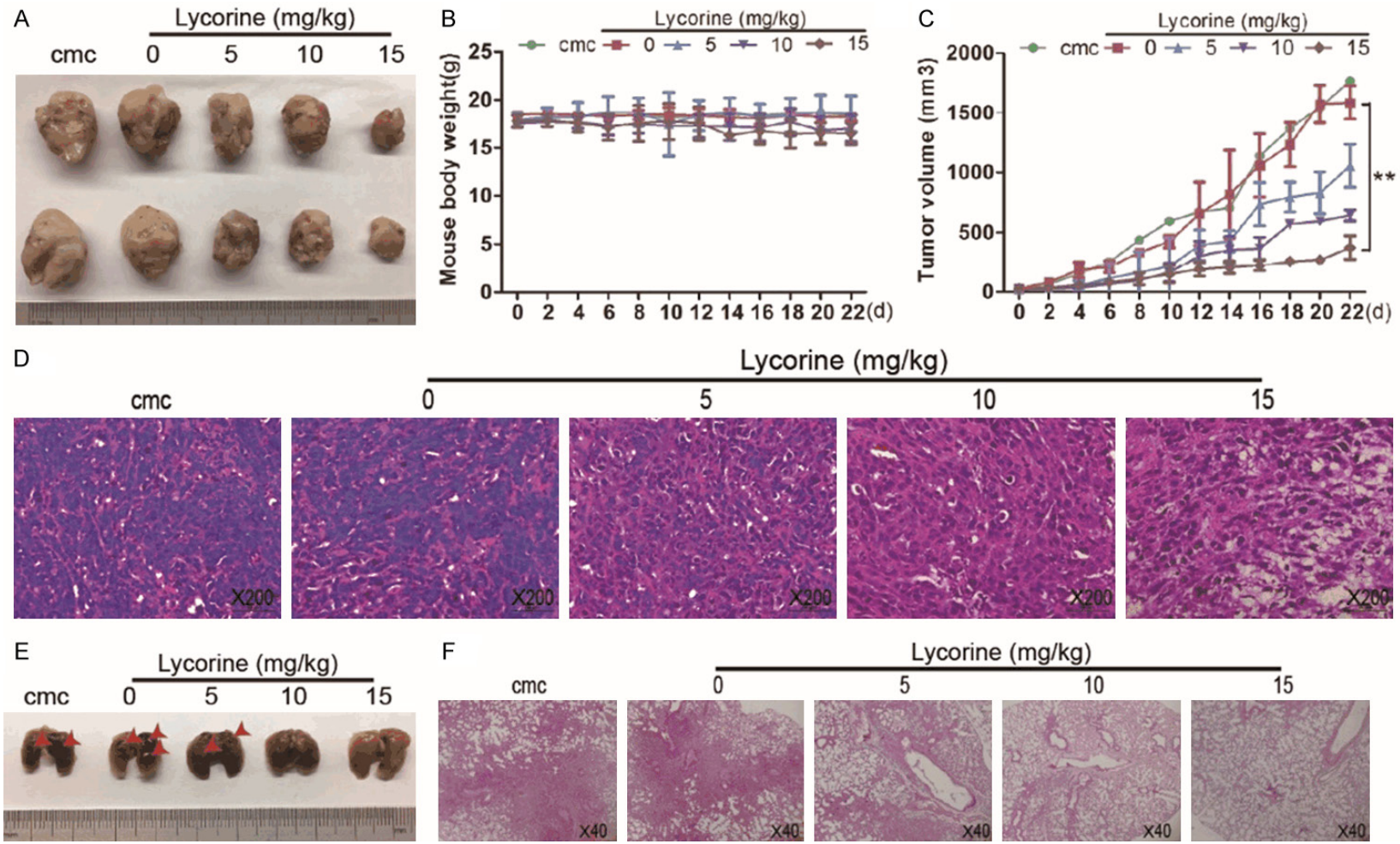
Lycorine inhibits OS cells growth and metastasis in orthotopic tumor model

Our above results have demonstrated that lycorine inhibited the growth and metastasis of OS cells *in vitro*. Next, we sought to validate the anti-OS activity of lycorine in a nude mice orthotopic model *in vivo*. Although the body weight of each lycorine group showed no obvious difference compared with that of control group (**Figure 6B**), the average tumor volume in lycorine treatment group was much smaller than that of control group (**Figure 6A** and **6C**). The retrieved tumor sample was then analyzed by H&E staining, and we noticed that lycorine treatment decreased OS proliferation and aggression, reduced nucleo/plasm ratio, and caused cell nucleus pycnosis and nuclear cracking (**Figure 6D**). Therefore, these results imply that lycorine may inhibit the growth of OS *in vivo*. Furthermore, we obtained pulmonary tissues from sacrificed nude mice, we found that pulmonary metastasis focuses of 143B OS cells was also obviously reduced by lycorine (**Figure 6E** and **6F**). Moreover, consistence with our *in vitro* results, the IHC staining results indicated that lycorine suppressed the level of PCNA, N-cadherin, Bcl-2, β-catenin, p-ERK1/2(T202/Y204) and p-AKT(S473) in orthotopic OS tissues (**Figure 6G-L**). To sum up, these results further validate that lycorine inhibit the growth and metastasis of OS cells in orthotopic tumor model *in vivo*.

Discussion

OS is the most common primary bone malignancies characterized by high tumor heterogeneity, poor prognosis and high lung metastases [18]. In the recent decades, clinical surgery and chemotherapy have improved the 5-year survival of OS patients. The common-used anti-OS chemotherapy drugs are MTX, ADM and IFO, however, these drugs have two main defects: (i) Strong toxic side effect against normal cells. (ii) Be prone to developing drug resistance. Increasing evidences have implied that natural products extracted from herbal plants are probably to be potential resources of anti-cancer drugs for their high efficiency to cancer cells and low toxicity against normal cells [11, 19, 20]. Lycorine, a alkaloids in the bulbs of plant lycoris, has been reported to effectively inhibited several type of cancers [21]. In this study, we decided to investigate the anti-cancer effects of lycorine on the proliferation, apoptosis, migration and invasion of OS cells *in vitro* and *in vivo.* We found that lycorine inhibited proliferation, promoted apoptosis, prevented migration and invasion possibly via suppression of Wnt/β-catenin, ERK1/2 and PI3K/AKT signaling pathway.

Agents that inhibit tumor cell proliferation and migration can be used to retard cancer progression and improve survival rate [22]. In the current study, we validated that lycorine effectively reduced the cell proliferation of 143B,


A
Lycorine (mg/kg)
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B
Lycorine (mg/kg)
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Mouse body weight(g)
0 2 4 6 8 10 12 14 16 18 20 22(d)
C
Lycorine (mg/kg)
cmc
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Tumor volume (mm3)
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D
Lycorine (mg/kg)
cmc
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Lycorine (mg/kg)
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F
Lycorine (mg/kg)
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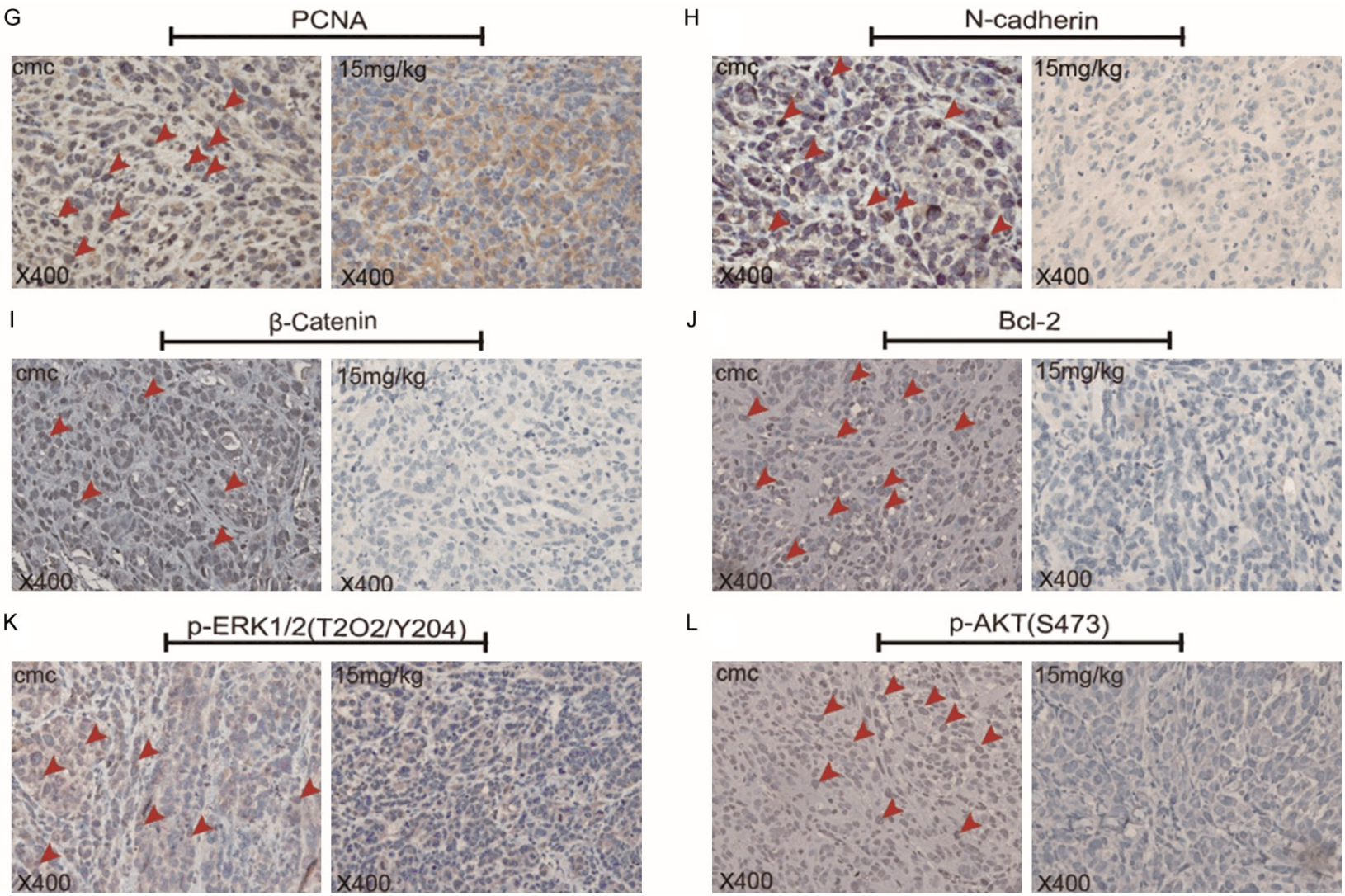

Figure 6. Lycorine inhibits growth and metastasis of 143B OS cells in orthotopic tumor model *in vivo*. A. Lycorine inhibited the growth of 143B OS cells *in vivo*. B. Lycorine had no obvious effect on the body weight of nude mice. C. Lycorine inhibited the tumor volumes compared with controls. D. Lycorine inhibited the proliferation of 143B OS cells in orthotopic tumor model (H&E staining, ×200). E. Lycorine inhibited the pulmonary metastasis of orthotopic OS *in vivo*. F. Lycorine reduced the pulmonary metastatic of 143B OS cells in orthotopic tumor model (H&E staining, ×40). G-L. Lycorine inhibited the level of PCNA, N-cadherin, Bcl-2, β-catenin, p-ERK1/2 (T202/Y204), p-AKT (S473) of 143B OS cells in orthotopic tumor model (IHC staining, ×400). Data are presented as the means ± SD, n = 3, *P<0.05, **P<0.01.

MG63, SaoS2, U2OS OS cells, and inhibited colony formation ability of 143B and MG63 cells. It is noteworthy that although lycorine exerted strong growth-inhibitory effect on OS cells, it displayed relative lower toxicity against human normal cells MIHA, HK-2, HEB and HS-5. These results imply us that lycorine is probably a safe and effective drug candidate for the treatment of OS. Cell cycle is a physiological process, and the disorder of cell cycle transition is one of the important reasons for tumor formation. Cyclins are a group of proteins which control cell cycle and initiate DNA synthesis, for example, CyclinD1 and CyclinE1 are both vital regulatory factors of G1/S phase cell cycle progression [23-25]. It has been previously reported that lycorine effectively inhibited mitotic proliferation of human ovarian cancer cells through inducing cell cycle arrest at the G2/M transition [10]. In the current study, we demonstrated that lycorine triggered G1/S phase arrest and significantly reduced the expression level of CyclinD1 and CyclinE1, which are well-established G1 phase transition markers. Therefore, these data indicate that lycorine-induced G1/S arrest of OS cells may be correlated with the reduction of CyclinD1 and CyclinE1 protein.

We next investigated the effect of lycorine on apoptosis of OS cells. The extrinsic mitochondrial pathway and intrinsic death receptor pathway are two important cell apoptosis pathways. The death receptor pathway is triggered by the bind of Fas-associated protein with death domain (Fadd) to its' activated FAS receptor, and subsequently activates Caspase8 to form the death induction signal complex. Activated caspase8 may not only activate Caspase3, but also cut Bid and induce activation of mitochondrial apoptotic pathway [26]. In mitochondrial apoptotic pathway, Cytc release from mitochondria to form the apoptosome and activate Caspase9. Then, Caspase9 further enhances the cleavage of PARP, and results in cell apoptosis [27]. Therefore, Fadd and Caspase8 are important markers of death receptor pathway while Caspase9 is a classical marker of mitochondrial apoptotic pathway. Bcl-2 can decrease the release of Cytc to suppress cell apoptosis through binding with Bax. In cell apoptosis process, Bcl-2 is anti-apoptotic molecule while Bax is pro-apoptotic. In the current study, we documented that lycorine treatment promoted apoptosis of OS cells by Hoechst33258 staining and flow cytometry analysis. The activation of apoptosis was further confirmed by western blot that the protein level of c-Caspase3, c-PARP and Bax was increased, while Bcl-2 was decreased. Our results imply that the extrinsic mitochondrial pathway and the intrinsic death receptor pathway are both activated by lycorine treatment.

Epithelial mesenchymal transition (EMT) and matrix metalloproteinases (MMPs) have been considered as important mechanisms through which to enhance tumor cell migration and invasion [28-32]. EMT promotes cytoskeletal reorganization in epithelial cells and down-regulates the proteins that maintain epithelial cell-cell junctions [33-36]. Here, we demonstrated that lycorine increased the protein level of epithelial marker E-cadherin, whereas reduced the protein level of interstitial markers N-cadherin, Vimentin, and Snail. These findings indicate that EMT process in OS cells is probably reversed by lycorine. MMP-2, MMP-7 and MMP-9 are the most important member of MMPs which can hydrolyze extracellular matrix including collagen laminin, fibronectin and elastin to promote tumor cell migration and invasion [37]. Indeed, it has been documented that Six1 in macrophages facilitated HCC cell invasion by up-regulating MMP-9 [37, 38]. Similarly, MMP-2 and MMP-7 have been reported to play important roles in cancer invasion and metastasis [26]. Our results clarified that lycorine treatment significantly decreased the protein level of MMP-2, MMP-7 and MMP-9 in 143B OS cells. Collectively, our results suggest that lycorine may prevent migration and invasion through reversing EMT and inhibiting the protein level of MMP-2, MMP-7 and MMP-9.

The Wnt/β-catenin signaling belongs to the canonical Wnt pathway that causes an accumulation of β-catenin and its eventual translocation into the nucleus. Wnt/β-catenin pathway was shown to be abnormal activated in various cancers and has been implicated in the tumor progression [39], recurrence [27], metastases [40] and chemotherapy resistance [41]. It has been reported by previous studies that Wnt/β-catenin signaling plays positive role in the development of breast cancer [42], ovarian cancer [43], and prostate cancer [44]. A systems biology analysis of genomic and proteomic profiles

revealed that Wnt/β-catenin signaling activation enhanced the metastasis of OS [45, 46]. Our data in this current study showed that lycorine inhibited the activation of Wnt/β-catenin signaling pathway. The member of MAPK family such as c-Jun N-terminal kinase (JNKs), extracellular signal-regulated kinase (ERK1/2), and p38 play critical roles in maintaining cell survival and apoptosis [47]. Previous study has demonstrated that Amentoflavone inhibited metastatic potential through suppression of ERK1/2 activation in U2OS OS cells [48]. Ursolic acid was found to trigger apoptosis in human OS cells via activation of caspase and ERK1/2 pathway [49]. Here, our western blot results testified that lycorine significantly inhibited the phosphorylation level of ERK1/2, while there was no significant alteration in the protein level of total level of ERK1/2. We also employed western blot to detect the effect of lycorine on the phosphorylation of p38 and JNK, and found no obvious change (data not shown). In addition, western blot results showed that lycorine significantly inhibited PI3K/AKT pathway which has been proved to play a key role in the development of OS [50]. Although our results provided strong evidence for a role of Wnt/β-catenin, ERK1/2 and PI3K/AKT signal pathway in OS cells, other pathways cannot be ruled out. Thus, our findings imply that lycorine may suppress OS cells growth and metastasis through inhibiting classical Wnt/β-catenin, ERK1/2 and PI3K/AKT signaling pathway.

In summary, we find that lycorine inhibits the proliferation, induces apoptosis and suppresses migration and invasion of OS cells *in vivo* and *in vitro*. Mechanically, in addition to the classic Wnt/β-catenin signaling pathway, the ERK1/2/MAPK and PI3K/AKT signaling pathway may play a role in the inhibitory lycorine on OS cells. This study further confirms that lycorine not only has good curative effect on OS, but also has relative lower toxicity against normal cells. Our findings may imply the clinical potential of lycorine to become an efficacious therapeutic drug candidate for the treatment of OS.

Disclosure of conflict of interest

None.

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