# MS4322 is a selective protein arginine methyltransferase 5 degrader with antitumor effects in cervical cancer cells

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**Abstract.** Drug therapy serves a key role in the treatment of cervical cancer, which is one of the most common types of solid tumor in female patients. Therefore, it is important to seek more effective and less toxic therapies. Protein arginine methyltransferase 5 (PRMT5) is a key oncogenic target in cervical cancer, providing a rational basis for the development of targeted therapeutic agents. MS4322 is a highly selective proteolysis targeting chimera degrader specifically targeting PRMT5. Therefore, the present study aimed to investigate the therapeutic potential of MS4322 against cervical cancer and the underlying molecular mechanisms. The effects of MS4322 on human cervical HeLa cells were investigated by Cell Counting Kit-8, clone formation, wound healing and Transwell assay, flow cytometry, immunofluorescence staining, immunohistochemistry and small interfering RNA assay. PRMT5 expression was upregulated in cervical cancer tissue, and functional analyses confirmed that PRMT5 promoted the proliferation of cervical cancer cells. MS4322 significantly decreased PRMT5 mRNA expression, as well as the proliferation, migration, invasion and clone formation ability of HeLa cells, leading to cell cycle arrest in G0/G1 phase and inducing apoptosis. Mechanistically, MS4322 downregulated the expression of PRMT5, β-catenin, Wnt-3a, and c-myc, while upregulating GSK-3β, thereby inactivating the Wnt/β-catenin pathway. These findings indicated that MS4322 exerted anti-tumor effects via regulating the PRMT5/Wnt/β-catenin pathway and may serve as a promising candidate agent for cervical cancer treatment.

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## Introduction

Cervical cancer is a major global health burden, with an estimated 342,000 patients dying from the disease annually (1). The overall prognosis of patients with cervical cancer is poor due to recurrence and metastasis (2). Cervical cancer is a complex process that involves multiple links, stages and genes and the molecular mechanisms of cervical cancer are unclear (3). Thus, a deeper understanding of the underlying molecular mechanisms governing cervical cancer development and progression is key to devising novel preventive strategies and targeted therapeutic interventions.

Protein arginine methyltransferases (PRMTs) catalyze the methylation of guanidinium nitrogen atoms of arginine residues, using S-adenosylmethionine as the methyl donor (4). In eukaryotic organisms, methylated arginine exists in three primary forms: Monomethylarginine, asymmetric dimethylarginine and symmetric dimethylarginine (SDMA) (5). To date, nine PRMT family members have been identified, which are classified into three different types based on their catalytic activity. Type I PRMTs mediate asymmetric dimethylation of the two ω-nitrogen atoms on the arginine side chain, while type II PRMTs catalyze symmetric dimethylation of the same residues. A third subgroup comprises monomethyltransferases, which introduce a single methyl group onto arginine residues (6). Notably, an additional type IV PRMT class has been characterized in fungi, which generates δ-Ng-methylarginine (7). Accumulating evidence in recent years has underscored the key role of PRMT-mediated arginine methylation in multiple hallmarks of tumorigenesis, including cancer development, metastasis and chemoresistance (8,9).

As a key type II protein arginine methyltransferase, protein arginine methyltransferase 5 (PRMT5) catalyzes the symmetric dimethylation of histone residues including H2R3, H4R3, H3R2 and H2R8, thereby participating in the epigenetic

regulation of gene transcription (7). PRMT5 also mediates the methylation of non-histone proteins substrates such as the transcription factors P21, P53 and Enhancer of Zeste Homolog 2 (10). In the fetal period, PRMT5 is highly expressed in the ovary, while it is lowly expressed in other tissue and organs, and shows low expression in all organs in adults, suggesting high PRMT5 expression is closely related to the development of a number of tumors (11). Literature reports that high PRMT5 expression is associated with poor prognosis in triple-negative breast cancer (12). PRMT5 is upregulated in colorectal cancer and promotes tumor formation (13,14). PRMT5 may serve as a potential target for the treatment of cervical cancer and the development of PRMT5-specific inhibitors or degraders in combination with chemotherapeutic agents as therapeutic agents for cervical cancer is becoming a popular study (15,16).

Studies have confirmed that multiple signaling pathways are involved in tumorigenesis, including AKT/PI3K, RAS/ERK, mTORC and NF-κB signaling pathways (17-19). Wnt/β-catenin signaling controls key embryonic and somatic cellular processes, such as determining cell fate, organogenesis and tissue homeostasis. It contributes to pathological conditions including inflammatory disorder and metabolic diseases, and is critically involved in cancer development. Abnormal activation of Wnt/β-catenin signaling is associated with many aspects of cancer progression, malignant transformation and poor prognosis (20). The Wnt/β-catenin signaling pathway serves an important role in the pathogenesis of cervical cancer (21). Activation of Wnt signaling promotes cervical cancer cell proliferation, migration, invasion, cell cycle progression and resistance to apoptosis (22). Therefore, the Wnt/β-catenin signaling pathway is considered an important target for the treatment of cervical cancer.

MS4322 is a first-in-class, highly selective proteolysis targeting chimera specifically targeting PRMT5, which was developed via protein hydrolysis-targeted chimeric technology by conjugating the PRMT5 inhibitor EPZ015666 with Von Hippel-Lindau (VHL) E3 ligase ligand (S,R,S)-AHPC-Me (VHL-2) (23). Notably, MS4322 decreases PRMT5 expression in breast cancer MCF7 cells in a concentration-, time-, VHL- and proteasome-dependent manner and exhibits favorable plasma exposure in mouse pharmacokinetic studies (23). Further studies have revealed that MS4322 treatment also leads to a significant decrease in PRMT5 protein expression and robust inhibition of cell proliferation in multiple other cancer cell lines, including A549 (lung adenocarcinoma), A172 (glioblastoma), and Jurkat (leukemia) cells (24-26).

In summary, PRMT5 is highly expressed in a variety of malignant tumors, and its high expression is associated with malignant tumor progression and patient prognosis. To the best of our knowledge, it has not been reported whether MS4322 has antitumor effects on cervical cancer cells. Therefore, the present study focused on whether PRMT5 is highly expressed in cervical cancer tissue and has a tumor-promoting effect, as well as the *in vitro* antitumor effect of MS4322.

## Materials and methods

*Chemicals and cell lines*. MS4322 (purity, >95%) was synthesized by Tianjin Gao Tan Technology Co. MS4322 was prepared as 90 mM master mix with DMSO and stored at -20˚C. Human cervical cancer HeLa cells were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were incubated at 37˚C in DMEM with 10% (vol/vol) FBS and antibiotics (penicillin 100 U/ml and streptomycin 10 $\mu$g/ml; all Gibco (Thermo Fisher Scientific, Inc.).

*Clinical samples*. A total of three female patients diagnosed with cervical cancer were enrolled at Linyi People's Hospital (Linyi, China). Clinical samples, including cancerous and adjacent non-cancerous tissues (>2 cm from the tumor margin) were collected. Sample collection was conducted from June 2023 to August 2023. The patient ages ranged from 52 to 68 years, with a median age of 60 years. A total of one patient was classified as well-differentiated (G1), one as moderately differentiated (G2) and one as poorly differentiated (G3); based on the International Federation of Gynecology and Obstetrics (FIGO) 2021 staging system, one patient was at stage IB2, one at stage IIA1 and one at stage IIA2 (27). All patients had no history of other malignant tumors or severe chronic disease before enrollment, and their preoperative vital signs were stable. The inclusion criteria were as follows: Histopathologically confirmed cervical cancer with complete pathological data; and complete clinical medical records containing key information. The exclusion criteria were as follows: Concurrent *in situ* malignant tumors at other sites; severe dysfunction of vital organs including the heart, liver or kidney; uncontrolled infection; previous exposure to any anti-tumor therapies; and incomplete medical records or data that preclude data analysis. The present study was approved by the Ethics Committee of Linyi People's Hospital, Linyi, China; approval no. 202306-H-093) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants prior to sample collection.

*Cell Counting Kit (CCK)-8 assay*. Cells ($3x10^3$/well) were seeded in 96-well plate and incubated at 37˚C overnight. The culture medium was replaced with fresh complete medium (Gibco; Thermo Fisher Scientific), and cells were treated with MS4322 (20, 40, 60, 80, 100, 120, 140 and 160 $\mu$M) at 37˚C for 24, 48 and 72 h followed by addition of 20 $\mu$l 10 $\mu$M CCK-8 solution (Beyotime Biotechnology) at 37˚C for 4 h. Cell viability was calculated as follows: [(Absorbance of experimental well)-(absorbance of blank well)]/[(absorbance of control well)-(absorbance of blank well)] x100%. Absorbance at 450 nm was measured using a microplate reader (EPOCH-SN; Bio Tek Instruments, Inc.).

*Colony formation assay*. HeLa cells were plated in 6-well plates at 350 cells/well and treated with 120 $\mu$M MS4322 at 37˚C, and the medium was changed every 3 days. After 14 days at 37˚C, cells were washed twice with PBS at room temperature, fixed in 75% ethanol on ice for 30 min and then stained with 0.5% crystal violet at room temperature for 30 min. A colony was defined as a cell cluster consisting of >50 cells. Colonies were counted manually under an inverted light microscope. Clone formation rate was calculated as follows: Number of clones/number of inoculated cells x100%. Each experiment was repeated three times.

*Wound healing assay*. Hela cells were cultured to 90-100% confluence in 6-well plates. The cell monolayer was scraped with a 10 $\mu$l pipette tip to produce a wound, washed with PBS to remove detached cells and the cells were incubated in serum-free DMEM (Gibco; Thermo Fisher Scientific, Inc.). Cells were incubated at 37˚C for 24 h. Photographs were taken at 0 and 24 h using a Nikon inverted light microscope (Nikon Instruments). Wound width analysis was performed with Image-Pro Plus 6.0 software (Media Cybernetics, Inc.). Data are shown as the mean of three independent experiments.

*Migration and invasion assay*. For cell migration, $2.5x10^4$ cells in 100 $\mu$l serum-free DMEM (Gibco, Thermo Fisher Scientific) were seed into the upper compartment of Transwell inserts in 6-well plates. The upper chamber was filled with serum-free DMEM. The lower chamber was filled with DMEM containing 10% FBS (Gibco; Thermo Fisher Scientific). Cells were incubated at 37˚C for 72 h, then fixed with 4% paraformaldehyde for 5 min at room temperature. Following washing with PBS three times, cells were stained with 0.5% crystal violet blue at room temperature for 5 min, then washed with double-distilled water. Cells on the upper surface of the insert were removed with a cotton swab. The positively stained cells were examined under an inverted light microscope. For the cell invasion assay, Transwell inserts were precoated with Matrigel (Corning) at 37˚C for 4 h to allow gelation.

*Cell transfection*. The PRMT5-small interfering RNA: Forward: 5'-GCCCAGUUUGAGAUGCCUU-3' and reverse, 5'-CGGUCAAACUCUACGGAA-3' and negative control: Forward: 5'-UUCUCCGAACGUGUCACG U-3' and reverse, 5'-ACGUGACACGUUCGGAGAA-3' (HANBIO Biotechnology). The PRMT5 overexpression plasmid (OE-PRMT5) and PRMT5-small interfering RNA (siRNA) were purchased from HANBIO Biotechnology Co., Ltd. HeLa cells were seeded in culture plates at a density of $1.85x10^4$ cells/$cm^2$ and transfected at 70-80% confluence. The PRMT5 overexpression plasmid, empty vector, PRMT5 or negative control siRNA were transfected into HeLa cells using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. For plasmid transfection, 4 $\mu$g of plasmid DNA was used per well of a 6-well plate. For siRNA transfection, 1 $\mu$g of siRNA was used per well of a 6-well plate. Transfection was performed at 37 for 6 h, after which the cells were cultured for 24-48 h at 37˚C in a 5% $CO_2$ incubator and then harvested for subsequent experiments.

*Cell apoptosis HeLa cells were harvested and co-stained with Annexin V-fluorescein isothiocyanate and propidium iodide using the Annexin V apoptosis detection kit (cat*. no. 556547; BD Biosciences), according to the manufacturer's instructions. The apoptotic cells were analyzed by flow cytometry (Accuri C6; BD Biosciences) using CellQuest Pro software, v3.3 (Becton, Dickinson and Company). Apoptosis rate was calculated as follows: (early apoptotic cells + late apoptotic cells)/total cells x100%

*Cell cycle assay*. The concentration of HeLa cells was adjusted to $2.5x10^5$ cells/ml using DMEM and seeded into 6-well plates. HeLa cells were treated with 120 $\mu$M MS4322 at 37˚C for 3 days. Cells were collected and washed twice with PBS and 700 $\mu$l pre-cooled 80% ethanol was added to give a final ethanol concentration of 70%. Cells were fixed at 4˚C for >4 h, then washed twice again with PBS. A total of 100 $\mu$l RNase (50 $\mu$g/ml) and 50 $\mu$g/ml propidium iodide (50 $\mu$g/ml) were added in the dark at room temperature for 30 min. Cell cycle distribution was analyzed by flow cytometry using a BD FACSCalibur (BD Biosciences). Data were analyzed using FlowJo software (version 10.6.2, BD Biosciences) Untreated cells were used as the control group. Each experiment was repeated three times.

*RNA extraction and reverse transcription-quantitative (RT-q) PCR*. RNA was extracted using TRIzol (Thermo Fisher Scientific, Inc.) and RT was performed using a PrimeScript RT Reagent kit (Takara Bio, Inc.) at 37˚C for 15 min, followed by heat inactivation at 85˚C for 5 sec. The qPCR reaction system consisted of 10 $\mu$l SYBR Green Master Mix (Thermo Fisher Scientific, Inc.), 0.4 $\mu$l forward primer, 0.4 $\mu$l reverse primer, 0.4 $\mu$l 50X ROX Reference Dye 2 (Thermo Fisher Scientific, Inc.), and 4.8 $\mu$l double-distilled water. Amplification was performed using a real-time PCR system under the following conditions: initial denaturation at 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 5 sec and annealing at 60˚C 30 sec. The relative expression of target genes was calculated by $2^{-\Delta\Delta Cq}$ method (28) using GAPDH as the internal reference gene. Each experiment was repeated three times. The primer sequences were as follows: PRMT5: Forward, 5'-TGACCAATAAGAAGGGAT-3' and reverse, 5'-GGCATT AGGTGGAGGAC-3' and GAPDH: Forward, 5'-TCAAGA AGGTGGTGAAGCAGG-3' and reverse, 5'-TCAAAGGTG GAGGAGTGGGT-3'.

*Protein extraction and western blotting*. Cells were collected, washed twice with PBS, lysed with PARP buffer (Beyotime Biotechnology) and centrifuged at 14,800 x g at 4˚C for 5 min. The protein concentration was determined via a BCA Protein Assay kit (Beyotime Biotechnology). Proteins (20 $\mu$g/lane) were separated using 10% SDS-PAGE and transferred to a PVDF membrane, which was blocked with 5% non-fat milk in TBST (0.1% Tween-20) for 1 h at room temperature. Membranes were then incubated with primary antibodies at 4˚C overnight. The membrane antibodies included PRMT5 (cat. no. ab109451; 1:1,000; Abcam), E-cadherin (cat. no. ab231303; 1:1,000; Abcam), snail (cat. no. ab167609; 1:1,000; Abcam), Vimentin (cat. no. ab92547; 1:1,000; Abcam), Bax (cat. no. ab32503; 1:1,000; Abcam), Bcl-2 (cat. no. ab194583; 1:1,000; Abcam), GSK-3β (cat. no. ab32391; 1:1,000; Abcam), C-myc (cat. no. ab32072; 1:1,000; Abcam), Wnt-3a (cat. no. Ab219412; 1:1,000; Abcam) and GAPDH (cat. no. ab181602; 1:5,000; all Abcam). Subsequent incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (cat. no. SA00001-2; 1:3,000; Wuhan Sanying) at room temperature for 1 h. Immunoreactive bands were visualized using an enhanced chemiluminescence kit (Beyotime Biotechnology). Images were scanned using a chemiluminescence and fluorescence imaging system (Hangzhou Shenhua Technology Co., Ltd.) and analyzed using Image-Pro plus software (Image-Pro plus 5.1, Media Cybernetics, Inc.). GAPDH was used as an internal control to regulate protein loading.

*Immunohistochemistry (IHC)*. The samples were fixed with 10% formalin at room temperature for 24 h and embedded in paraffin. The paraffin-embedded samples were cut into 4-$\mu$m-thick sections. The prepared tissue sections were subjected to IHC staining for the detection of PRMT5 protein. The samples were baked at 60˚C for 3 h to enhance tissue adherence. Next, the sections were deparaffinized in xylene and rehydrated through a graded series of ethanol solution. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide at room temperature for 10 min. Antigen retrieval was performed by incubating the sections in EDTA buffer (cat. no. ZLI-9072; OriGene) and heating in a microwave oven for 13-15 min. After blocking with 5% goat serum (cat. no. DD13; OriGene) at room temperature for 30 min, the sections were incubated with primary antibody against PRMT5 (cat. no. ab109451; 1:200; Abcam) overnight at 4˚C. Following four washes with PBS, the sections were incubated with horseradish peroxidase-conjugated goat anti-rabbit/mouse secondary antibody (cat. no. PV-9000; 1:1,000; ZSGB Biotechnology) at 37˚C for 30 min. The sections were washed four times with PBS again. Finally, the sections were developed with DAB at room temperature for 3-5 min, followed by counterstaining with Mayer's hematoxylin at room temperature for 1-2 min. All staining and morphological observations were performed using a light microscope. Image acquisition and quantitative analysis of IHC were performed using ImageJ software (Version 1.53k, National Institutes of Health).

*Immunofluorescence detection*. Following incubation with 120 $\mu$M MS4322 for 72 h at 37˚C in a humidified incubator with 5% $CO_2$, HeLa cells were fixed with 70% methanol at room temperature for 10 min and blocked with PBS containing 10% BSA (Thermo Fisher Scientific, Inc.) at room temperature for 30 min. Cells were incubated with primary antibodies overnight at 4˚C. The primary antibodies were as follows: E-cadherin (cat. no. ab231303; 1:200; Abcam), Snail (cat. no. ab167609; 1:200; Abcam), and Vimentin (cat. no. ab92547; 1:200; Abcam). After primary antibody incubation, cells were washed three with PBS. Subsequently, cells were incubated with Cy3-labeled anti-rabbit secondary antibody (cat. no. ab6939; 1:400; Abcam) at room temperature for 1 h and stained with DAPI (2.0 $\mu$g/ml) at room temperature for 5 min. Fluorescence signals were detected using an inverted fluorescence microscope (cat. no. DM505, Nikon Co., Ltd.).

*Statistical analysis*. All quantitative data were obtained from at least three independent biological replicates and are presented as mean ± standard deviation (SD). Comparisons between two independent groups were performed using the unpaired Student's t-test, while comparisons among >2 groups were conducted via one-way ANOVA followed by Tukey's post hoc test; for data that violated variance homogeneity, Dunnett's T3 test was employed instead. All statistical analyses were carried out using SPSS 20.0 (IBM Corp.) and GraphPad Prism 9.0 software (GraphPad Software Inc.; Dotmatics). $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*PRMT5 is highly expressed in cervical cancer tissue*. PRMT5 has been documented to be overexpressed in multiple types of malignancy, including breast (12) and colorectal cancer (13). To investigate the expression profile of PRMT5 in cervical cancer and their matched adjacent tissue, IHC was performed to detect PRMT5 expression in paired tissue samples derived from three patients. PRMT5 exhibited significantly elevated expression in cervical cancer tissues compared with adjacent tissue (Fig. 1).

*PRMT5 overexpression promotes HeLa cell migration and invasion, while knockdown of PRMT5 inhibits HeLa cell migration and invasion*. To elucidate the functional role of PRMT5 in cervical cancer cells, the present study generated stable HeLa cell lines with PRMT5 overexpression (Fig. 2A) and knockdown (Fig. 3A).

CCK-8 and colony formation assay revealed that stable PRMT5 overexpression significantly enhanced cell proliferation and colony-forming capacity relative to control cells (Fig. 2B and C). Transwell migration and invasion assays further indicated that PRMT5-overexpressing HeLa cells exhibited a significant increase in migratory and invasive potential (Fig. 2D and E). In addition, flow cytometry cell cycle analysis showed that compared with control cells, PRMT5-overexpressing HeLa cells displayed a decreased proportion of cells in the G0/G1 and S phases, accompanied by an increase in the G2/M phase fraction (Fig. 2F). These PRMT5-mediated pro-tumorigenic effects were abrogated following treatment with MS4322.

Conversely, PRMT5 knockdown in HeLa cells resulted in a significant inhibition of cell proliferation and colony-forming ability (Fig. 3B and C). Consistently, Transwell assay demonstrated that migratory and invasive capacity were significantly impaired in PRMT5-knockdown HeLa cells (Fig. 3D and E). Flow cytometric analysis further confirmed that PRMT5 knockdown induced a cell cycle arrest characterized by an increased proportion of cells in the G0/G1 phase and a decrease in the S and G2/M phases (Fig. 3F).

*MS4322 inhibits cervical cancer HeLa cell viability in vitro*. HeLa cells were treated with MS4322 and the cell viability was detected by the CCK-8 assay (Fig. 4A). MS4322 significantly inhibited the viability of HeLa cells *in vitro* in a concentration- and time-dependent manner. The inhibitory effect was not obvious when the concentration was <120 $\mu$M and at 24 and 48 h. Therefore, MS4322 at 120 $\mu$M and 72 h were selected for the subsequent experiments. The half-maximal inhibitory concentration of MS4322 on HeLa cells was 170.408 $\mu$M. Compared with the control group, the number of colonies formed by HeLa cells was reduced after 72 h MS4322 treatment (Fig. 4B), indicating that MS4322 had a significant inhibitory effect on the colony formation ability of HeLa tumor cells (Fig. 4C).

*MS4322 inhibits the migration and invasion of HeLa cells*. Cell migration plays a key role in a variety of cellular physiopathological processes, including tumor metastasis (29). The present study examined the effect of MS4322 on HeLa

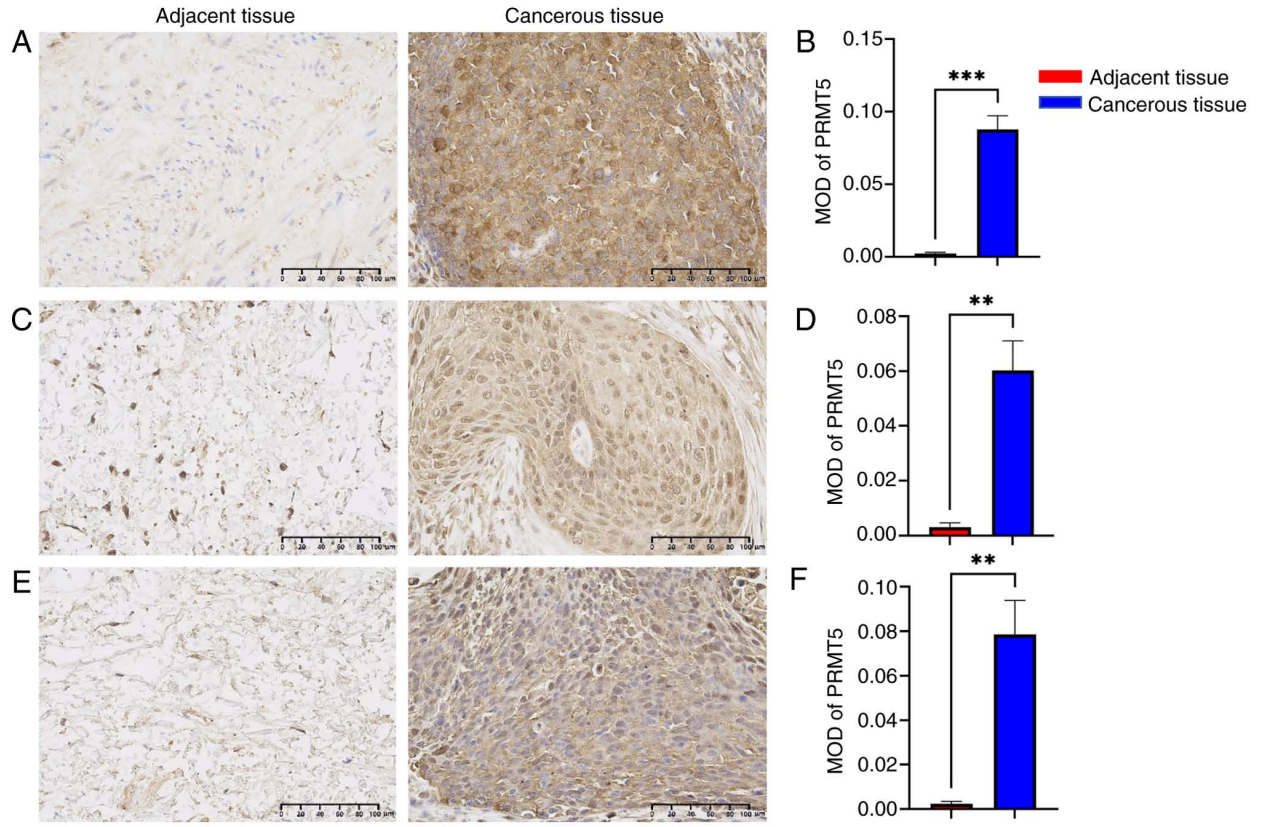


Figure 1. Immunohistochemical staining of PRMT5 expression in cervical cancer tissue. (A) IHC of PRMT5 in cervical tissues from patient 1 (magnification, x400). (B) MOD of PRMT5 expression in patient 1. (C) IHC of PRMT5 in cervical tissue and. (D) MOD of PRMT5 expression in patient 2. (E) IHC of PRMT5 in cervical tissues from patient 3 (magnification, x400). (F) MOD of PRMT5 expression in patient 3. **P<0.01; ***P<0.001. PRMT5, Protein arginine methyltransferase 5; MOD, mean optical density.

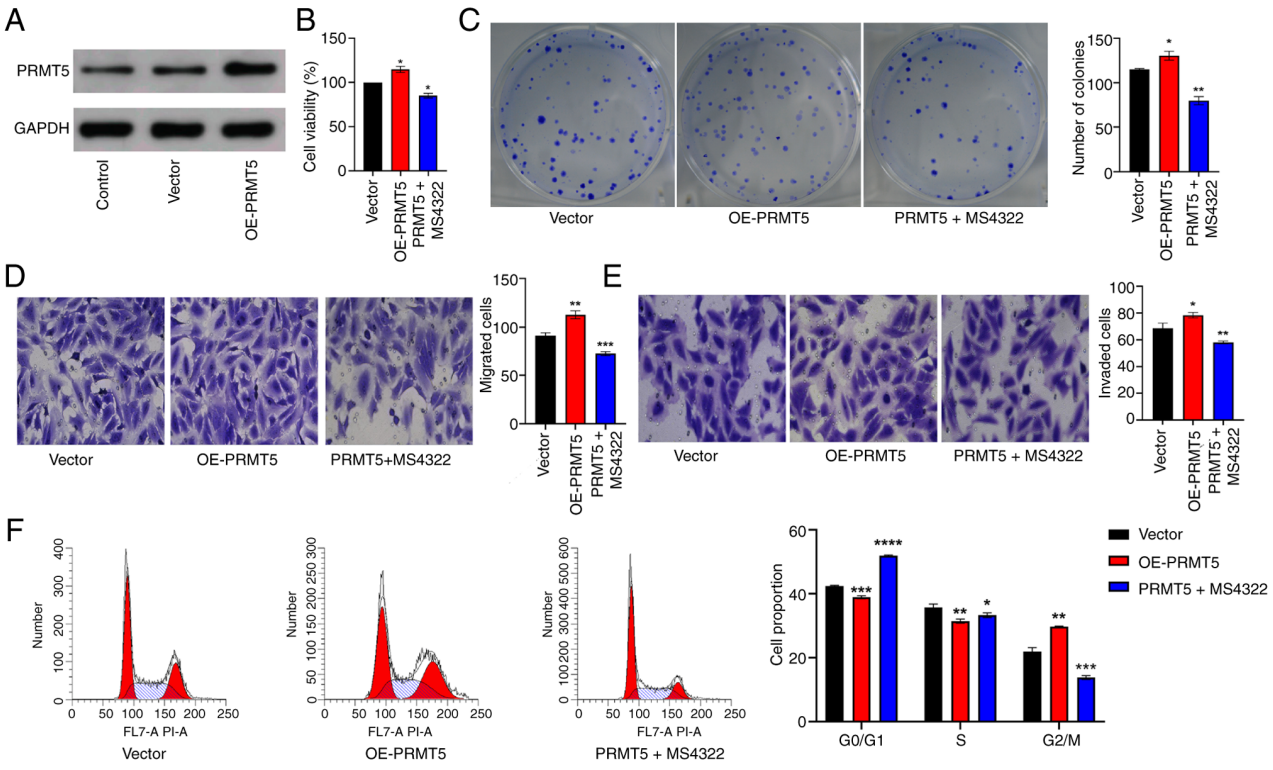


Figure 2. Effect of PRMT5 overexpression on the proliferation, migration and invasion of HeLa cells. (A) Western blotting demonstrated overexpression of PRMT5 in transfected HeLa cells. (B) Cell Counting Kit-8 assay demonstrating the cell proliferation. (C) Clone formation assay. Cell (D) migration and. (E) invasion rate determined by Transwell assay (magnification, x200). (F) Cell cycle analysis. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 vs. the control. PRMT5, Protein arginine methyltransferase 5; OE, overexpression.

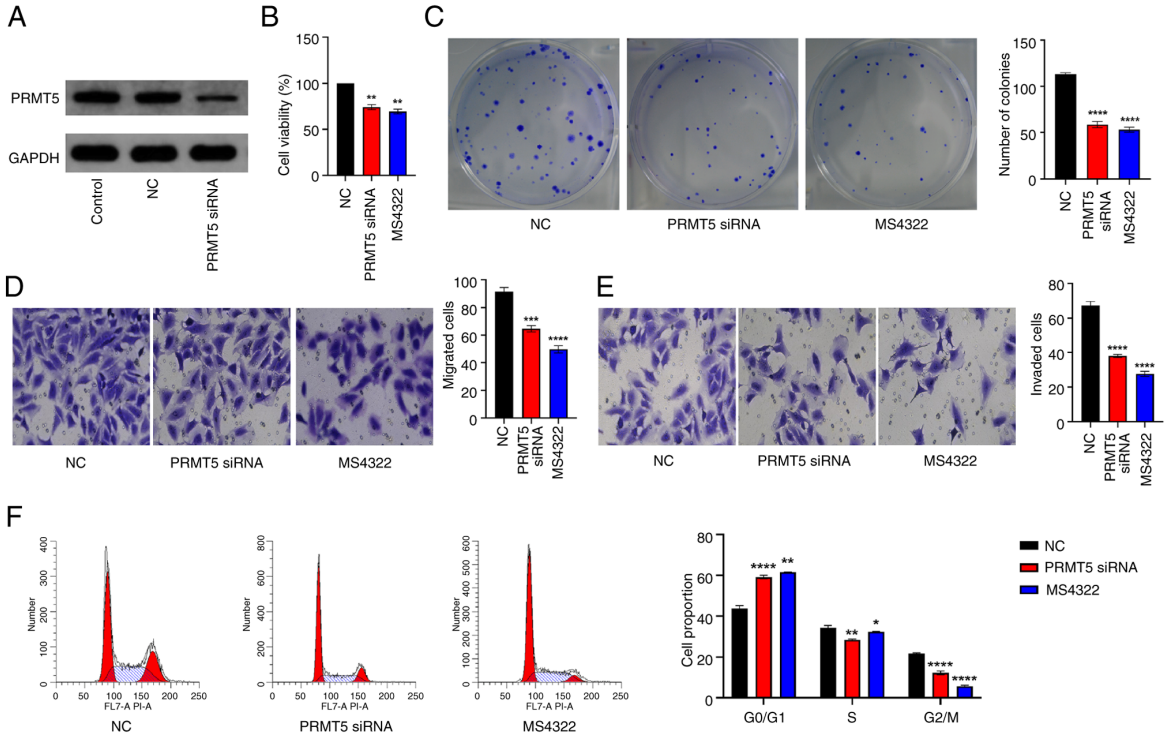


Figure 3. Inhibitory effect of PRMT5 knockdown on HeLa cell proliferation, migration and invasion. (A) Western blotting demonstrated knockdown of PRMT5 in transfected HeLa cells. (B) Cell Counting Kit-8 assay demonstrating the cell proliferation. (C) Clone formation assay. Cell (D) migration and (E) invasion rate determined by Transwell assay (magnification, x200). (F) Cell cycle analysis. $^{*}$P<0.05; $^{**}$P<0.01; $^{***}$P<0.001; $^{****}$P<0.0001 vs. the control. PRMT5, Protein arginine methyltransferase 5; si, small interfering; NC, negative control.

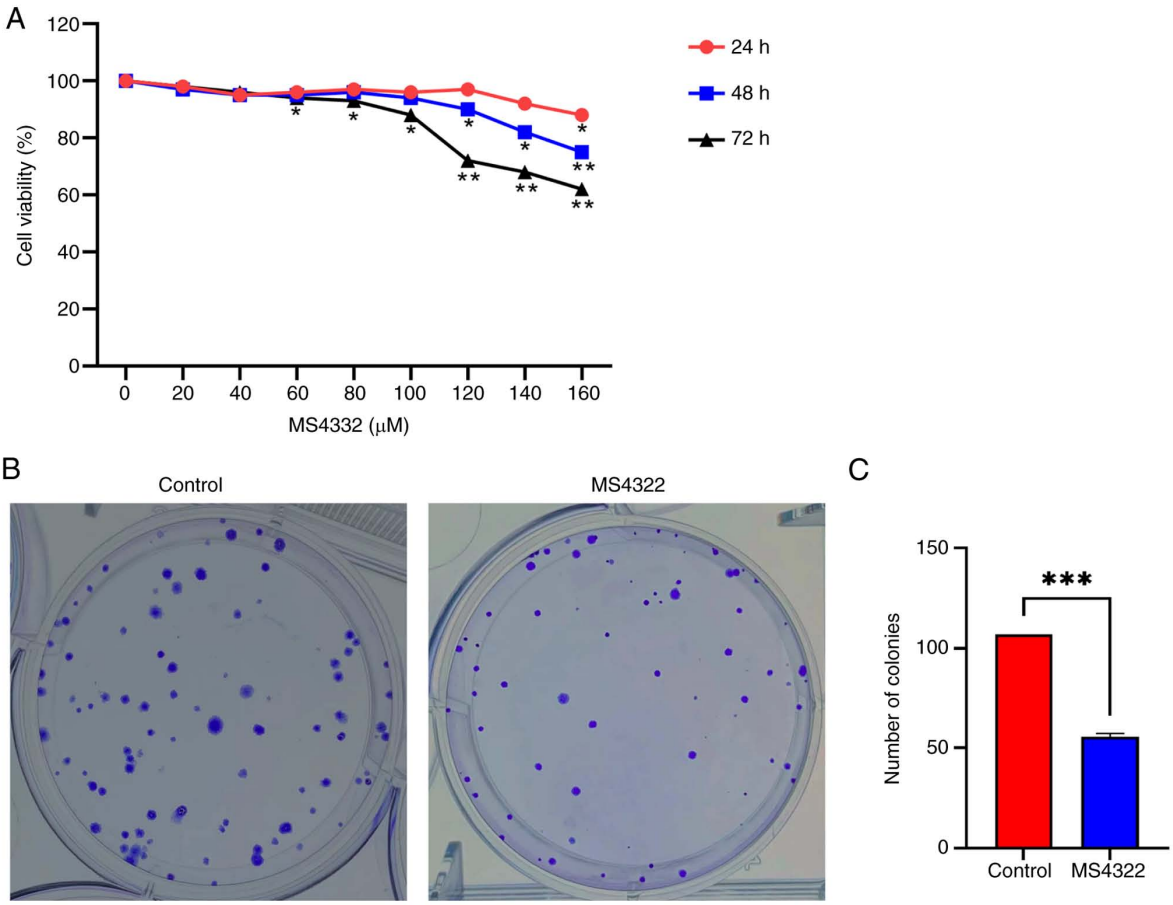


Figure 4. Effect of MS4322 on the viability of cervical cancer HeLa cells. Effect of MS4322 on (A) viability and (B) colony formation of HeLa cells. (C) Number of colonies. $^{*}$P<0.05; $^{**}$P<0.01; $^{***}$P<0.001 vs. control group.

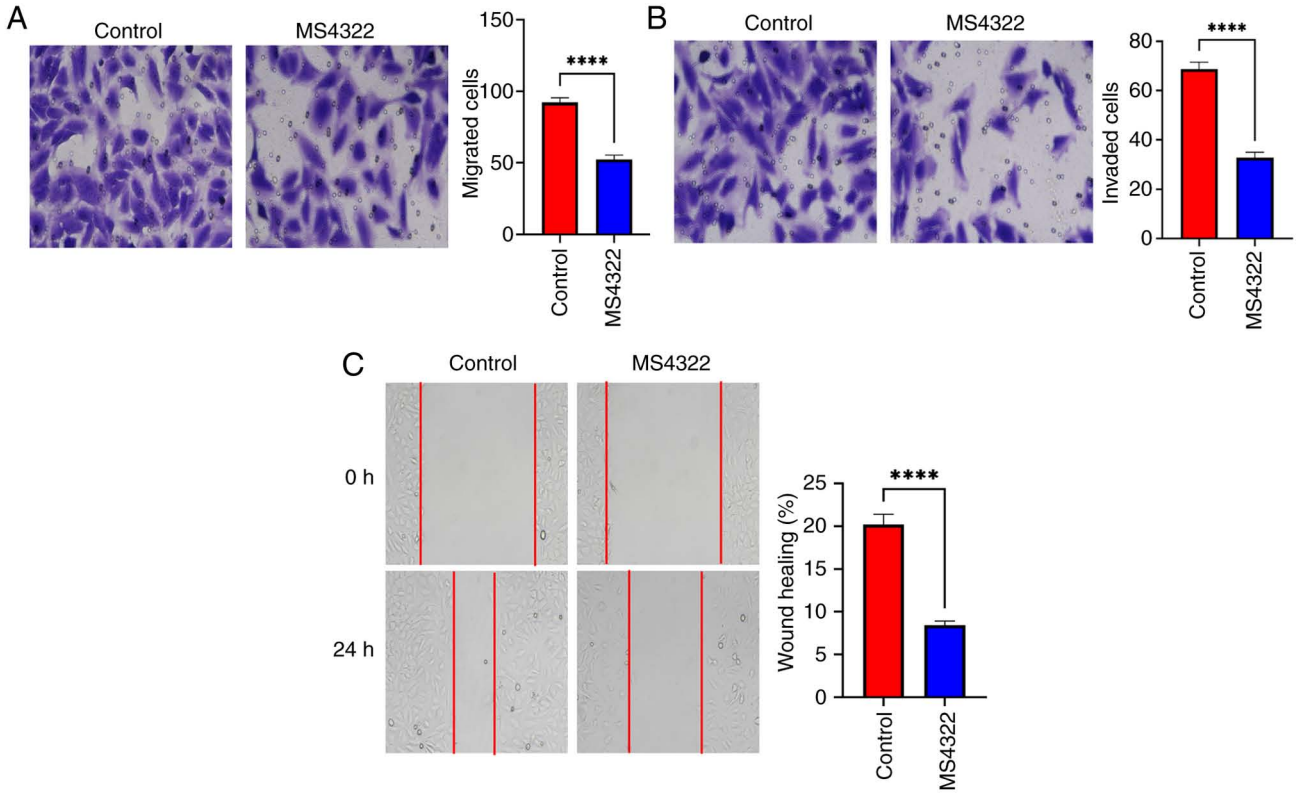


Figure 5. Effect of MS4322 on wound healing, migration and invasion of HeLa cells. (A) Migration and (B) invasion of HeLa cells using Transwell assay (magnification, x200). (C) Relative wound width. ****P<0.0001.

cell migration and invasion. The number of migrating HeLa cells was significantly decreased when treated with MS4322 compared with the control (Fig. 5A). In the wound healing assay, untreated HeLa cells migrated to fill the scratched area within 24 h, but MS4322 significantly prevented migration of HeLa cells (Fig. 5C). Taken together, these experiments confirmed that MS4322 inhibited the migration of HeLa cells. To investigate the effect of MS4322 on HeLa cell invasion, Matrigel assay was performed. Compared with the control group, the number of invaded HeLa cells was significantly decreased in the MS4322-treated group (Fig. 5B). These results indicated that MS4322 significantly inhibited the migration and invasion ability of HeLa cells *in vitro*.

*MS4322 inhibits epithelial-mesenchymal transition (EMT) in HeLa cells*. EMT is hypothesized to play an important function in tumor cell migration and invasion (30). Therefore, the present study examined the effect of MS4322 on the expression of EMT-associated proteins in HeLa cells. E-cadherin protein expression was significantly upregulated, while Snail and vimentin protein expression was significantly downregulated in HeLa cells treated with MS4322 (Fig. 5A and B). The fluorescence intensity of Snail and Vimentin was significantly weakened (Fig. 6D and E) and the fluorescence intensity of E-cadherin was significantly enhanced (Fig. 6C) after MS4322 treatment compared with the control group. These results suggested that the inhibitory effect of MS4322 on HeLa cell migration and invasion may be related to EMT.

*MS4322 induces apoptosis in HeLa cells*. Apoptosis serves an important role in both tumorigenesis and therapy (31). The number of Annexin V-positive cells significantly increased from 4.09 to 21.98% after 72 h MS4322 treatment, suggesting that MS4322 promoted apoptosis in HeLa cells (Fig. 7A). In addition, the expression of apoptosis-associated proteins was detected by western blotting following MS4322 treatment of HeLa cells. MS4322 significantly inhibited the expression of Bcl-2 protein and elevated the expression of Bax protein (Fig. 7B and C). These results suggested that MS4322 influenced the degree of apoptosis in cervical cancer HeLa cells.

*MS4322 induces G0/G1 phase arrest in HeLa cells*. MS4322 induced apoptosis in HeLa cells, and inhibition of cancer cell proliferation is usually associated with cell cycle arrest. To investigate the potential mechanism by which MS4322 inhibited the proliferation of HeLa cells, the present study analyzed the effect of MS4322 on the cell cycle by flow cytometry. The proportions of untreated HeLa cells in G0/G1, G2/M and S phase were 43.33, 34.91 and 21.76%, respectively; however, the proportions of HeLa cells in G0/G1, G2/M and S phases were 61.63, 33.18 and 5.19%, respectively, when treated with MS4322 (Fig. 8A). The proportion of cells in G0/G1 phase was significantly increased, and the proportion of cells in G2/M and S phases was significantly decreased following treatment, which indicated that MS4322 arrested cells in G0/G1 phase (Fig. 8B).

*MS4322 inhibits Wnt/β-catenin pathway activity in cervical cancer cells*. The Wnt/β-catenin pathway serves a key role in several biological processes, including cell proliferation,

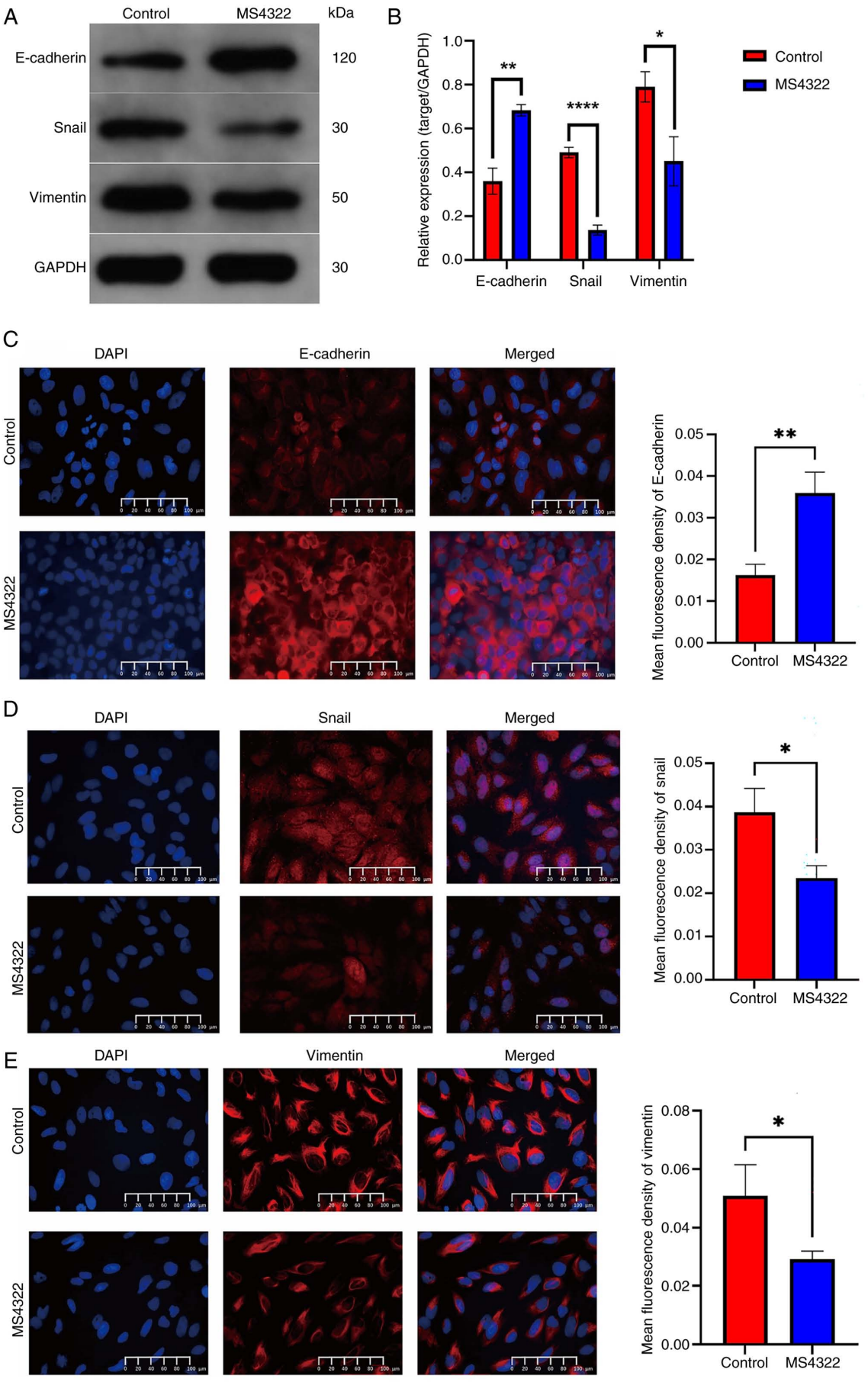


Figure 6. Effect of MS4322 on EMT in HeLa cells. (A) Western blot analysis of (B) EMT-associated protein following MS4322 treatment. Immunofluorescence detection of expression of (C) E-cadherin, (D) Snail and (E) vimentin following MS4322 treatment. $^{*}$P<0.05; $^{**}$P<0.01; $^{****}$P<0.0001. EMT, epithelial-mesenchymal transition.

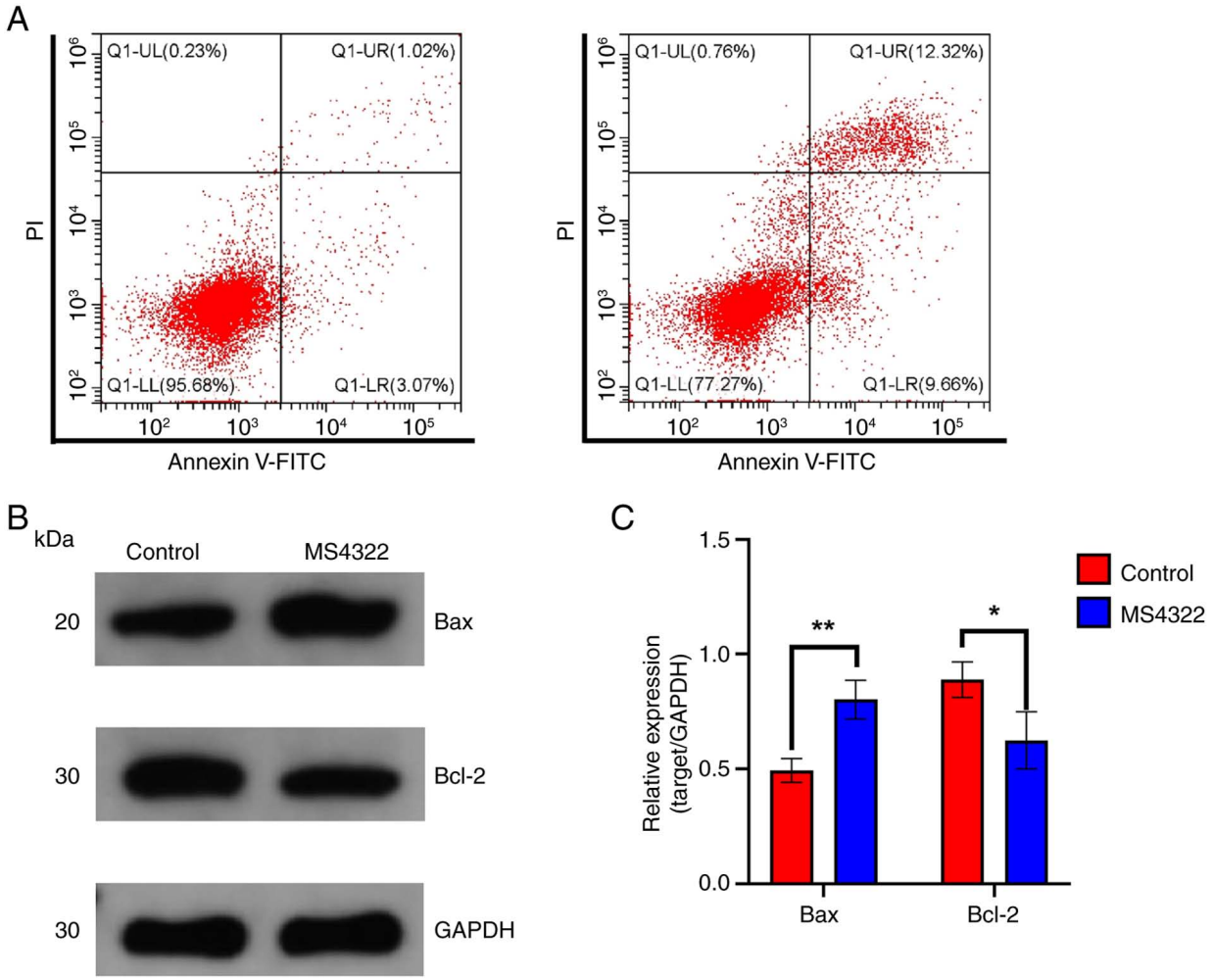


Figure 7. Effect of MS4322 on apoptosis of HeLa cells. (A) Annexin V/PI staining for cell apoptosis. (B) Western blot analysis of (C) Bax and Bcl-2 proteins after MS4322 treatment. *P<0.05; **P<0.01.

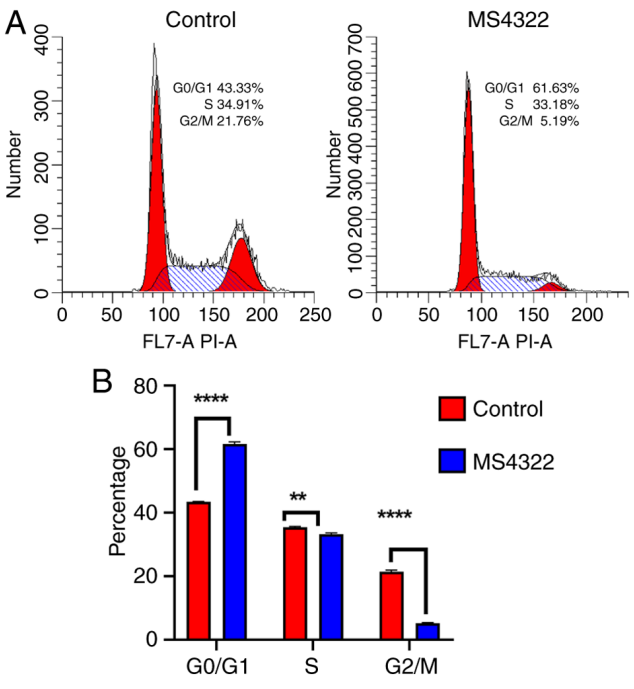


Figure 8. Effect of MS4322 on HeLa cell cycle progression. (A) Flow cytometry analysis of (B) cell cycle distribution. **P<0.01, ****P<0.0001.

differentiation, migration and apoptosis (32). β-catenin is the key molecule of the pathway, and its stability and nuclear translocation are key steps in the activation of the pathway. Wnt-3a, a member of the Wnt family, activates the pathway, while glycogen synthase kinase 3β (GSK-3β), a negative regulator of the pathway, inhibits pathway activation by phosphorylating β-catenin and promoting its degradation (20,21).

Decreased expression of β-catenin and Wnt-3a protein, as well as increased expression of GSK-3β following MS4322 treatment suggested that MS4322 may inhibit the activation of the Wnt/β-catenin pathway. In addition, the expression of c-myc, a target protein downstream of the Wnt/β-catenin pathway, was also significantly inhibited (Fig. 9).

## Discussion

Cervical cancer is the fourth most common female malignant tumor in the world, with ~660,000 new cases and 350,000 deaths globally in 2022 (33). It is the second most prevalent cancer in developing countries, accounting for 94% of global cervical cancer deaths and with an incidence 2-3 times higher than that in developed countries (34). Notably, its incidence is on the rise globally, with a 50.1% increase in cases, and the age of onset tends to be younger (35). Epidemiological

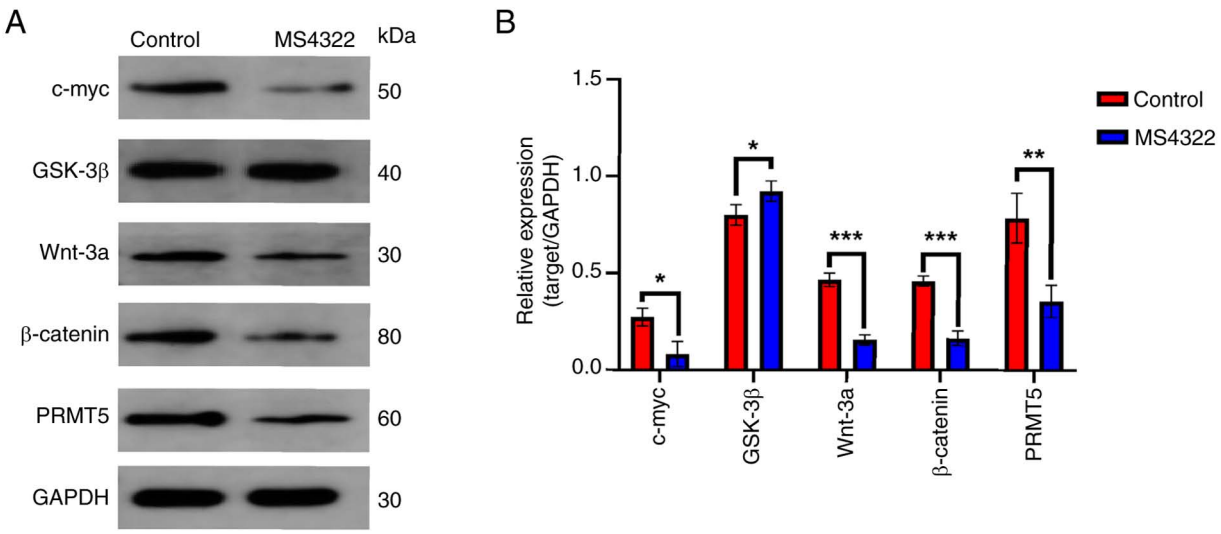


Figure 9. Effect of MS4322 on the expression of Wnt/β-catenin pathway-related protein. (A) Western blot analysis of (B) c-myc, GSK-3β, Wnt-3a, β-catenin and PRMT5 proteins following MS4322 treatment of HeLa. $^{*}$P<0.05; $^{**}$P<0.01; $^{***}$P<0.001. GSK-3β, glycogen synthase kinase 3β; PRMT5, Protein arginine methyltransferase 5.

studies have shown that HeLa cells are associated with human papillomavirus (HPV) infection and defective immune function (36,37). Early cervical cancer has a high cure rate, with a 5-year survival rate >90% for FIGO Stage I, while advanced and recurrent cervical cancer has a poor prognosis, with a 5-year survival rate of only 10-20% (38). Cervical cancer cell proliferation, metastasis and drug resistance are notable problems in clinical treatment. Therefore, clarifying the specific mechanisms underlying proliferation, recurrence and metastasis of cervical cancer and searching for effective therapeutic targets are key to improve treatment prospects.

Arginine methylation is one of the most common types of post-translational modification of proteins in mammalian cells. PRMTs are notable regulators of epigenetically mediated gene expression, which modulates a variety of processes, including mRNA splicing, DNA damage response, stem cell function, TGF-β and EMT signaling pathways and immune response (39). Dysregulation of the expression of PRMTs leads to cancer recurrence, decreases patient survival and induces drug resistance by modulating the TGF-β, PI3K/Akt and MAPK/ERK signaling pathways (40-42). Numerous PRMTs are aberrantly expressed in cancer cells and promote cancer stem cell generation, EMT and tumor cell proliferation. The development of small molecules targeting PRMTs has led to the generation of chemical probes for the modulation of most PRMTs and treatment based on targeting PRMT1 and PRMT5 (43). PRMT5 is a key member of the type II PRMT family. It contains an inter-terminal domain that binds methylated epitope protein 50, which is essential for its full methyltransferase activity. Its C-terminal domain harbors the methyltransferase catalytic motifs required for plasma membrane binding (44). PRMT5 serves a key role in normal cellular processes by catalyzing monomethylation and symmetric dimethylation of a range of histone and non-histone substrates, and is also an oncoprotein that epigenetically regulates the expression of certain oncogenes (45). For example, PRMT5 mediates the symmetric dimethylation of histone substrates such as H4R3 and H3R8 to epigenetically silence tumor suppressor genes (46). It also targets multiple non-histone substrates, including KEAP1 (9,12), SMAD4 (42), and AKT (46), thereby modulating the NRF2/HMOX1, TGF-β (42), and PI3K/AKT (46) signaling pathways to drive cancer cell proliferation, metastasis and immunotherapy resistance. Clinical studies (4,11) have shown that PRMT5 is highly expressed in numerous types of solid tumor and hematological malignancies and is associated with cancer development and progression. In addition, increased PRMT5 expression is associated with poorer overall survival and tumor metastasis (46,47). In ovarian cancer cells, PRMT5 gene expression is significantly upregulated, and this high expression is associated with poor prognosis. Mechanistically, PRMT5 enhances glycolytic flux and promotes the proliferation of ovarian cancer cells by regulating SDMA-mediated activity of enolase 1 activity (48). PRMT5 is highly expressed in lung cancer tissue and is associated with the progression of lung tumors and patient survival; PRMT5 affects lung cancer growth by promoting EMT and metastasis via hypoxia-inducible factor 1α/VEGFR/Akt/endothelial nitric oxide synthase (eNOS) signaling and promoting angiogenesis (49). Therefore, PRMT5 is becoming a promising anticancer target and has received great attention from the pharmaceutical industry and academia (50,51).

The present study demonstrated that PRMT5 was significantly upregulated in human cervical cancer compared with adjacent normal tissue. Functional experiments in HeLa cells revealed that PRMT5 overexpression markedly promoted cell proliferation, migration, invasion and colony formation, whereas PRMT5 knockdown exerted the opposite effects. The molecular mechanism by which PRMT5 induces HeLa cell proliferation and migration primarily involves the Wnt/β-catenin signaling pathway. PRMT5 upregulates Wnt-3a and β-catenin expression, thereby activating the downstream target gene c-myc to promote cell cycle progression and EMT (52). PRMT5 also suppresses apoptosis by upregulating the anti-apoptotic protein Bcl-2 and downregulating the pro-apoptotic protein Bax (53,54). At the phenotypic

level, PRMT5 decreases the epithelial marker E-cadherin and increases the mesenchymal markers vimentin and Snail, thereby facilitating migration and invasion (52).

MS4322 is a highly selective PRMT5 degrader and the first reported degrader targeting any PRMT family member. It is constructed by conjugating the PRMT5 inhibitor EPZ015666 to VHL E3 ligase ligand (S,R,S)-AHPC-Me (VHL-2) (44). MS4322 decreases PRMT5 expression in various cancer cell lines in a concentration-, time-, VHL- and proteasome-dependent manner and effectively suppresses cancer cell proliferation (44). The present study demonstrated that MS4322 significantly suppressed the viability, migration, invasion and colony formation of HeLa cells. Mechanistically, MS4322 downregulates PRMT5 at both mRNA and protein levels, reverses PRMT5-mediated oncogenic signaling, induces G0/G1 cell cycle arrest, inhibits EMT by upregulating E-cadherin and downregulating vimentin and snail, and promotes apoptosis by increasing Bax and decreasing Bcl-2 expression. Moreover, MS4322 suppresses the Wnt/β-catenin pathway by degrading PRMT5, downregulating Wnt-3a, β-catenin and c-myc while enhancing GSK-3β expression. Collectively, MS4322 targets PRMT5 to disrupt the Wnt-β-catenin-EMT axis, thereby inhibiting proliferation, migration and invasion and promoting apoptosis in HeLa cells.

EMT is regarded as a key pathological process in tumor progression. In the malignant evolution of tumors, EMT confers stronger invasive and metastatic abilities, and may allow tumor cells to escape apoptosis induced by chemotherapeutic agents, death receptor ligands and immune surveillance, which increases the difficulty of treatment and the mortality rate of patients (55). The present study demonstrated EMT-associated protein changes following MS4322 treatment: MS4322 upregulated the expression of E-cadherin protein and downregulated the expression of Snail and vimentin protein in HeLa cells.

Apoptosis is a tightly regulated and evolutionarily conserved program of cell death that serves a key role in life activities. It not only serves a key role in normal physiological processes such as embryogenesis and adult tissue homeostasis, but is also known for its role as a tumor suppressor mechanism (56). The role of apoptosis in cancer has received widespread attention for its importance in maintaining body homeostasis and preventing cancer development (9,15,18). However, when apoptosis is inhibited or resisted, it confers a survival advantage to cancer cells, promotes tumor evolution and growth and may lead to therapeutic failure (31,56,57). The Bcl-2 family is associated with tumor progression and Bcl-2/Bax ratio (pro- and anti-apoptotic proteins, respectively) determines tumor cell survival or death (58). The present study confirmed that MS4322 inhibited the protein expression of Bcl-2 by increasing Bax protein expression in cervical cancer HeLa cells. Consistently, BIIB021, an orally active, fully synthetic and selective small-molecule inhibitor of heat shock protein 90, has been reported to decrease Bcl-2 and elevate Bax levels in human cervical cancer cells (59). Cell cycle abnormalities can cause a variety of diseases, including cancer, and the rapid multiplication of tumors depends on uncontrolled cell cycle progression (60). Following 72 h MS4322 treatment, the number of cells in S and G2/M phase was decreased and the cells were blocked in G0/G1 phase.

Aberrant activation of the Wnt/β-catenin signaling pathway occurs in numerous types of malignant tumor and is closely associated with cancer development and progression, as well as renewal capacity and multidifferentiation potential of tumor cells (61). Aberrant activation of β-catenin contributes to cancer development (62). Dysregulation of the Wnt/β-catenin pathway is associated with HPV-associated cervical cancer (63). The present study confirmed that MS4322 downregulated PRMT5 mRNA expression in cervical cancer HeLa cells and investigated its effect on the expression of proteins in the Wnt/β-catenin signaling pathway. To the best of our knowledge, the present study is the first to demonstrate that MS4322 enhanced GSK-3β protein expression. By contrast, β-catenin, Wnt3a and downstream proto-oncogene c-myc protein expression was significantly decreased.

The anticancer activity of MS4322 is not restricted to cervical cancer cells; prior studies have shown that this compound effectively decreases PRMT5 protein expression and inhibits proliferation in multiple cancer cell lines, including MCF7 (breast cancer), A549 (lung adenocarcinoma), A172 (glioblastoma), and Jurkat (leukemia) cells (23,24). However, the mechanism of MS4322 underlying inhibiting the activity of Wnt/β-catenin signaling pathway remains to be investigated. The present study was conducted using the HeLa cell line. Future studies should validate the function of PRMT5 and the anticancer efficacy of MS4322 in other cervical cancer cell models or primary cells, thereby enhancing the generalizability and clinical relevance of the findings. Nevertheless, the present study demonstrated the inhibitory effect of MS4322 on HeLa cells and its potential mechanisms, providing novel insight into the pathogenesis of cervical cancer and offering promising new targets and strategies for its clinical diagnosis and treatment.

In conclusion, the present study found that PRMT5 showed high expression in cervical cancer tissues, PRMT5 promoted the migration and invasion of HeLa cervical cancer cells, while knockdown of PRMT5 inhibited these malignant phenotypes and induced cell cycle arrest. Furthermore, the PRMT5 degrader MS4322 exerted anti-tumor effects by targeting PRMT5, thereby suppressing the malignant biological behaviors of cervical cancer HeLa cells *in vitro*.

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**Availability of data and materials**

The data generated in the present study may be requested from the corresponding author.

**Authors' contributions**

RL designed the study, performed experiments, and wrote the manuscript. JC performed experiments and data analysis. ZC

and SW contributed to data analysis and participated in data discussion. TL interpreted data interpretation, participated in data discussion, and critically revised the manuscript. YX conceived the study conception, experimental design, and critical revision of the manuscript. RL and YX confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

**Ethics approval and consent to participate**

The present study was approved by the Ethics Committee of Linyi People's Hospital, Linyi, China (approval no. 202306-H-093) and performed in accordance with the Declaration of Helsinki. All participants provided written informed consent prior to sample collection.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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