

Semaphorin 4D promotes the proliferation and metastasis of bladder cancer by activating the PI3K/AKT pathway

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Abstract

The present study aimed to investigate the role of semaphorin 4D (Sema4D) in bladder cancer cell proliferation and metastasis *in vivo* and *in vitro*. Effects of Sema4D modulation on cancer cell viability and clonogenic abilities were assessed by MTT assay and colony formation assay. Cell apoptosis, cell cycle analysis, transwell assays, and wound-healing assays were also assayed. A mouse model of bladder cancer was established to observe the tumorigenesis *in vivo*. Our data showed that Sema4D was 4-fold upregulated in clinical bladder cancer tissues relative to noncancerous ones and differentially expressed in bladder cancer cell lines. Knockdown of Sema4D in bladder cancer T24 and 5637 cells significantly decreased cell proliferation, clonogenic potential, and motility. On the contrary, overexpression of Sema4D in bladder cancer SV-HUC-1 cells significantly increased cell viability and motility. Concordantly, knockdown of Sema4D impaired while overexpression of Sema4D promoted bladder cancer cell growth rates in xenotransplanted mice. Cell cycle was arrested by modulation of Sema4D. Cell apoptotic rates and the mitochondrial membrane potentials were consistently increased upon knockdown of Sema4D in T24 cells and 5637 cells. Western blotting revealed that epithelial–mesenchymal transition was promoted by Sema4D. The PI3K/AKT pathway was activated upon Sema4D overexpression in SV-HUC-1 cells, while it was inactivated by knockdown of Sema4D in T24 cells. All these data suggest that Sema4D promotes cell proliferation and metastasis in bladder cancer *in vivo* and *in vitro*. The oncogenic behavior of Sema4D is achieved by activating the PI3K/AKT pathway.

Keywords

Semaphorin 4D, bladder cancer, proliferation, metastasis, PI3K/AKT signaling

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Introduction

Bladder cancer is a common urologic cancer and accounts for more than 16,000 deaths globally annually.¹ Although many advances in surgery and chemotherapy have been developed, the efficacies of current therapies are limited by a range of adverse effects, toxicity, and drug resistance. The chance of tumors returning in 5 years is 30%–44%. Therefore, the prognosis for patients with bladder cancer remains poor, and novel therapeutic strategies and more effective agents for advanced disease are urgently needed.

The semaphorins (Sema) and their receptors, plexins, represent a large protein family.² Semaphorin-plexin axis signals have been implicated in various processes, including loss of cell–cell contact, alterations in cell adhesion, regulation of lymphocyte activation, and development of

the heart and vasculature.³ There are over 40 semaphorins identified to date, of which semaphorin 4D (Sema4D) is an immune-related one and expressed by T-lymphocytes, eosinophils, and to a lesser extent by dendritic cells and B-lymphocytes. The involvement of Sema4D in human tumorigenesis has been gradually observed recently.

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Recently, a Sleeping Beauty forward genetic screen identified Sema4D in axonal guidance and validated Sema4D as one oncogene driving osteosarcoma development and metastasis.⁴ Overexpression of Sema4D has also been observed in colorectal carcinoma,⁵ non-small cell lung cancer,⁶ ovarian cancer,⁷ and breast cancer.² Aberrant expression of Sema4D has been shown to associate with poor outcomes. Antibody blockade of Sema4D has been shown to promote immunomodulatory therapies against tumors.^{8,9} These promising reports have greatly ignited our interest in investigating the detailed roles of Sema4D in other solid tumors such as bladder cancer.

This study aimed to investigate the detailed role of Sema4D in bladder cancer cell proliferation and metastasis. Since Sema4D was found in bladder cancer tissues, we collected 25 clinical bladder cancer tissues to verify the expression of Sema4D. Thereafter, the possible mechanisms of Sema4D in bladder cancer progression were systemically studied with *in vitro* and *in vivo* assays. This report presented substantial data to suggest that Sema4D might be a critical mediator of bladder cancer cell proliferation and metastasis and therapies against Sema4D should be promising with respect to bladder cancer treatment in clinic.

Methods

Human samples

A total of 25 patients with clinically diagnosed bladder cancer, who had undergone traditional surgeries in Ningbo Beilun District People's Hospital, were included in this study. Prior to the surgeries, no chemotherapy or radiotherapy was received by the patients. The cancerous and adjacent noncancerous tissues from each case were obtained with the patients' consent. The protocol was approved by the Ethics Committee of Ningbo Beilun District People's Hospital.

Immunohistochemistry analysis

Formalin-fixed, paraffin-embedded tumor tissues harvested from mouse model were processed for immunohistochemistry analysis. Briefly, 4- μ m-thick slides were deparaffinized, hydrated, antigen retrieved, rinsed with phosphate-buffered saline (PBS), and incubated with primary antibodies at 4°C overnight. The slides were then washed in PBS and incubated with horseradish peroxidase-containing secondary antibody (Santa Cruz Biotechnology, Dallas, TX) for 1 hour at room temperature. The slides were developed in 3,3-diaminobenzidine (FASTDAB tablets; Sigma-Aldrich, St. Louis, MO). Images were taken with an Aperio ScanScope CS scanner (Aperio, Vista, CA).

Cell lines and cell culture

Bladder cancer cell lines T24, 5637, SV-HUC-1, BIU-87, TCCSUP, and HT-1376 were commercially purchased from

the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco) in a humidified incubator containing 5% CO₂ at 37°C. Culture medium was replaced every other day unless otherwise stated.

RNA isolation and quantitative real-time PCR

Total RNA from clinical patients and cultured bladder cancer cells were extracted with TriZol reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions. The quality and quantity of RNA samples were determined by collecting the absorbance of OD₂₆₀ and OD₂₈₀ with Nanodrop 2000 (Invitrogen, Carlsbad, CA). cDNAs were reversely transcribed from 8 ng RNAs from each sample by PrimeScript Reverse Transcriptase (TaKaRa). Afterwards, real-time polymerase chain reaction (RT-PCR) was performed with the SYBR Green reagent (TaKaRa) with the ABI 7900 machine (ABI Company, Santa Ana, CA). The primers used in this study were as follows:

Sema4D: forward, 5'-GTCTTCAAAGAAGGGCAACAGG-3' reverse, 5'-GAGCATTTCAGTTCCTCGTG-3';

GAPDH: forward, 5'-GGGAAACTGTGGCGTGAT-3' reverse, 5'-GAGTGGGTGTCGCTGTTGA-3'.

GAPDH was used as the reference gene.

Western blot analysis

Cells were cultured at 37°C and transfected with specific shRNAs against Sema4D or Sema4D expressing plasmid. Forty-eight hours posttreatment, cell lysates were collected and protein concentrations were detected by the traditional BCA Kit (Beyotime, Nantong, China). A total of 50 μ g proteins were loaded onto a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred to the PVDF membrane (Millipore, Burlington, MA) by the electrophoretic transfer system (Bio-Rad, Hercules, CA). After blocking in 5% fat-free milk for 1 hour, the membrane was incubated with primary antibodies at 4°C overnight. Primary antibodies against Sema4D, CDC25C, Cyclin B1, CDC2, Snail, Vimentin, N-Cadherin, E-Cadherin, and GAPDH were purchased from Santa Cruz Biotechnology. The primary antibodies against p-PI3K, PI3K, p-AKT, and AKT were commercially from Cell Signaling Technology (Danvers, MA) and those against Bcl-xl, Caspase-9, cleaved caspase-9, Caspase-3, cleaved caspase-3, PARP, and cleaved PARP were purchased from Abcam (Cambridge, UK). On the second day, the membrane was washed with TBST and then incubated with secondary antibodies (Santa Cruz Biotechnology) for 1 hour. Visualizations of the signals were performed by enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, MA). Finally, the signals from each detected molecule

were collected and measured by a FluroChem machine and analyzed with Image J analysis software.

shRNA and expression plasmid transfection

Specific shRNAs against Sema4D were designed and synthesized by Sigma-Aldrich and the lentivirus containing this shRNA was packaged by Genepharma Co. (Shanghai, China). The expression plasmid was purchased from Addgene (Cambridge, MA) and amplified in our laboratory. Transfection was conducted using lipofectamine 2000 (Invitrogen) according to the protocols, while the lentivirus containing shRNAs was added into the culture medium directly. After 6 hours for plasmid transfection or 24 hours upon lentivirus infection, the culture media was replaced and the cells were incubated at 37°C for subsequent analysis.

Cell viability analysis

The MTT assays were performed in T24, 5637, SV-HUC-1 bladder cells to evaluate cell viabilities. Primary cultured cells were seeded into 96-well plates at a concentration of 5×10^4 /well. After transfection with shRNAs or expression plasmids, the MTT (0.5 mg/mL) reagent was dissolved in the culture medium and incubated at 37°C for an additional 4 hours. Then, dimethyl sulfoxide (DMSO) was included to dissolve the MTT-formazan product. The absorbance was collected by a microplate reader (Tecan, Männedorf, Switzerland) at a test wavelength of 570 nm.

Clonogenic assays

The ability of Sema4D to alter the stemness of bladder cancer cells in both monolayer (2D) and mammosphere (3D) cultures was evaluated through colony formation analysis by counting the numbers of the colonies that form after incubation. For both 2D and 3D assays, 150,000 cells per well were seeded in 6-well plates and incubated for 24 hours in complete culture media with 10% FBS. Twenty-four hours postseeding, culture media was replaced with fresh one and cells were treated with shSema4D or its expression plasmid for 48 hours. Cells were then collected by trypsinization and 100 treated cells were seeded onto each well in a new 6-well plate. For 2D culture, cells were incubated for an additional 14 days in growth medium supplied with 10% FBS. At the end of incubation, media was discarded and cells were fixed with the fixing solution (3.7% paraformaldehyde in 70% ethanol) for 10 minutes. Afterwards, cells were stained with 0.05% crystal violet for 20 minutes and then washed with PBS. For 3D mammosphere cultures, the cells were incubated in mammosphere media (serum-free, growth factor-enriched) for 7 days, after which the spheres were stained with MTT to improve visualization. A dissecting microscope was used

to quantify the stained colonies. The protocols were in accordance with a previous report.²⁰

Cell cycle analysis

Cell cycle analysis was performed to explore the role of Sema4D in bladder cancer cells. Briefly, T24, 5637, and SV-HUC-1 cells were enzymatically dissociated and low-speedily centrifuged for 5 minutes at 4°C. After washing with PBS 2 times, cells were fixed with 70% pre-cold ethanol and incubated overnight at 4°C. After that, cells were centrifuged and resuspended in a propidium iodide solution (50 μ g/mL). RNase (20 μ g/mL) was added to each well and samples were incubated at 37°C for 1 hour. The solution was immediately analyzed for DNA content by FACSaria II flow cytometer (BD Biosciences, San Jose, CA). Finally, the distribution of cells in each cell phase was analyzed with FlowJo software (Treestar, Ashland, OR).

Cell apoptosis analysis

Bladder cancer cells (2.5×10^5 cells/mL) were seeded in 10-cm culture dishes (Corning, Manassas, VA) and incubated overnight. Cells were treated with lentivirus containing Sema4D shRNA and annexin V-FITC/PI staining was performed with the FITC-Annexin V Apoptosis Detection Kit I (Beyotime) according to the manufacturer's protocols. Data of the staining were analyzed by flow cytometry assay with a FACS Calibur machine and CellQuest software (BD Biosciences).

Mitochondrial membrane potential ($\Delta\Psi_m$) detection

After transfected with the specific shRNA against Sema4D, T24 and 5637 cells were collected and washed twice with cold PBS. Cells were then incubated with rhodamine 123 (Sigma-Aldrich) for 40 minutes in the dark. Subsequently, the cells were washed twice with cold PBS and analyzed by flow cytometry. Cells with high $\Delta\Psi_m$ were considered survival and with low $\Delta\Psi_m$ apoptotic.

Wound-healing analysis

T24, 5637, and SV-HUC-1 cells were seeded into 6-well plates until they were primarily 90% confluent. The culture medium was replaced with serum-free medium and incubated for an additional 24 hours. A sterile 10 μ L pipette tip was used to mark a line in the monolayer of each well. The cells were then washed with PBS 3 times and allowed to grow in the serum-free medium for a further 24 hours. Afterwards, the wounds were observed under a Nikon (Tokyo, Japan) microscope. The width of the scratch in each group was measured at 0 and at 24 hours posttreatment, respectively. The cell migration in the

3 cell lines was calculated with the following formula: (cell free area at 0 hours – cell free area at 24 hours)/cell free area at 0 hours.

Transwell assays

Serum-free media conditioned by the indicated cells or transfected with shRNA/expressing plasmid were used to resuspend cells at a density of 10,000 cells/well. These cells were seeded into the upper chamber (Corning) in 24-well plates. Meanwhile, the lower chamber was filled with 600 μ L culture medium supplemented with 10% FBS. After 24 hours, the medium was discarded and the membrane (8 μ m pore) was fixed with pre-cold methanol for 5 minutes at room temperature. After staining with 0.05% crystal violet for 5 minutes, cells on the upper surface of the membrane were removed with cotton swabs carefully. The cells on the lower surface of the membrane were photographed and counted with a Nikon microscope at a magnification of 200 \times . For the invasive assays, the membrane was pre-coated with Matrigel (Corning) for 6 hours at 37°C and the images of invaded cells were also captured with the microscope.

In vivo studies

Six-week-old male athymic BALB/c nude mice were commercially purchased and maintained in special pathogen-free (SPF) condition. Mice were divided into 4 groups: scramble T24 group, shSema4D T24 group, mock 5637 group, and Sema4D-overexpressed 5637 group ($n = 6$ for each group). Prior to experiments, T24 cells were infected with the specific shRNA against Sema4D (shSema4D T24 group) or with a control shRNA (scramble T24 group), while 5637 cells were transfected with Sema4D expressing plasmid (Sema4D 5637 group) or an empty vector plasmid (mock 5637 group). Afterwards, the cells (5×10^6) were injected subcutaneously into the right flank of each mouse. Tumor dimensions of each mouse (length and width) were measured twice a week during the 4-week culture period. Tumor volumes (TV) were then calculated with the following formula: $TV = L \times W^2/2$. Finally, all mice were killed and bladder cancer tissues were dissected for subsequent analyses. The protocols were approved by the Ethical Committee of Animal Care in Ningbo Beilun District People's Hospital.

For lung colonization, 2.5×10^6 of T24 bladder cancer cells that were treated with shSema4D or scramble shRNA were injected into the tail vein of mice ($n = 10$ for each group). Each mouse was weighed daily for 4 weeks. At the study endpoint, all mice were killed. Each mouse was checked for lung colonization. Collected lungs were immediately fixed in a 10% formaldehyde solution for subsequent histologic analysis.

Hematoxylin & eosin staining and immunofluorescence

Formalin-fixed bladder cancer samples from mouse models were processed, embedded in paraffin, and made into 4- μ m slices. Sections were deparaffinized in xylene twice and rehydrated with a graded alcohol series. Slides were then stained with hematoxylin & eosin (H&E) by the standard protocols. Immunofluorescence was performed with the primary antibody against cleaved caspase-3 (Abcam, 1:100). The secondary antibodies were purchased from Santa Cruz Biotechnology. In the control group, primary antibody was replaced with non-specific immunoglobulin G (normal IgG). Slices were then visualized and photographed with a Nikon microscope equipped with a digital camera. The data were analyzed with CellSens Dimension 1 Software (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Statistical analysis

Data are presented as mean \pm SD. Each experiment that required statistics was performed in 3 independent replicates. Student *t* tests were conducted to compare mean values between groups and a *p* value of less than 0.05 was considered statistically significant.

Results

Sema4D is upregulated in clinical bladder cancer tissues and differentially expressed in bladder cancer cell lines

Initially, the transcription level of Sema4D was examined in 25 cases of bladder cancer. It was shown that the average mRNA level of Sema4D in the cancerous tissues was 4-fold increased relative to that in the adjacent noncancerous normal tissues (Figure 1A), suggesting the upregulation of Sema4D in bladder cancer tissues. Furthermore, it was observed that Sema4D was most highly expressed in T24, 5637, SV-HUC-1, and BIU-87 cell lines, and least expressed in TCCSUP and HT-1376 cell lines (Figure 1B). This observation suggested that Sema4D was differentially expressed in bladder cancer cells, and T24, 5637, and SV-HUC-1 were selected for subsequent analyses.

Sema4D promotes bladder cancer cell viability in vitro

In consideration of the expression profile of Sema4D in the bladder cancer cell lines, we used a specific shRNA against Sema4D (shSema4D) to knock down the expression of Sema4D in T24 cells and 5637 cells and an expression plasmid of Sema4D to increase the Sema4D level in SV-HUC-1 cells. As shown in Figure 2A, the specific shSema4D significantly depleted, while Sema4D overexpression increased

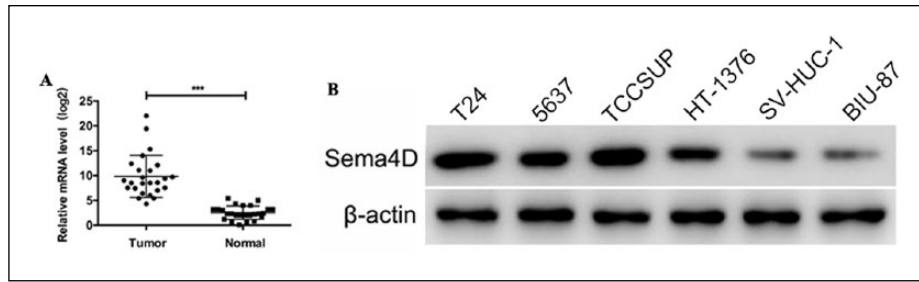


Figure 1. Sema4D is upregulated in clinical bladder cancer tissues and differentially expressed in bladder cancer cell lines. (A) The relative mRNA level of Sema4D in clinical bladder cancer tissues and in the adjacent noncancerous normal tissues; $n = 25$ for each. (B) The protein level of Sema4D in a series of bladder cancer cell lines was examined using Western blotting. $***p < .001$.

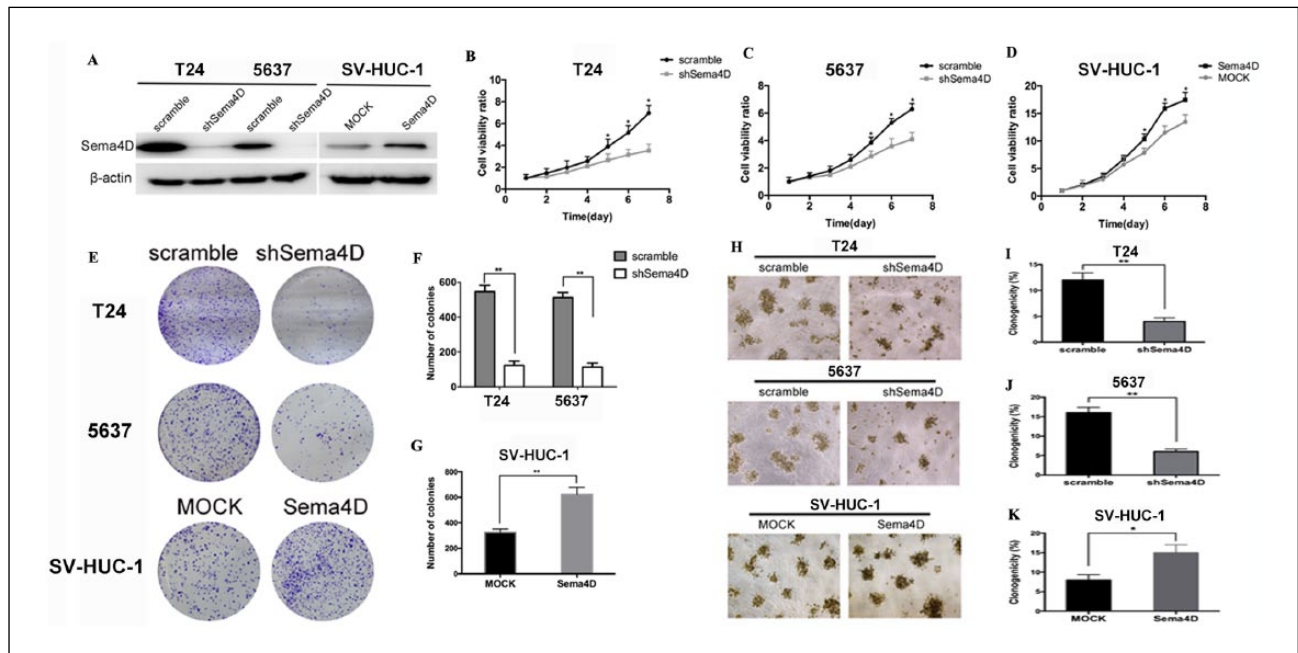


Figure 2. Sema4D promotes bladder cancer cell viability in vitro. (A) Specific shRNA against Sema4D (shSema4D) and an expression plasmid of Sema4D was used. Western blotting revealed the effectiveness of the shSema4D and expression plasmid. (B–D) MTT assay was performed to assess the effects of Sema4D knockdown in T24 cells and 5647 cells or Sema4D overexpression in SV-HUC-1 cells on the cell viability. (E–G) In the 2D colony formation assay, formed colonies were stained for visualization and quantified and shown in columns. (H–K) In the 3D cell cultures, the colonies formed by cells with each treatment were stained for visualization and quantified in columns. $*p < .05$; $**p < .01$.

the protein level of Sema4D. Next, in the cell viability assay, it was observed that knockdown of Sema4D significantly slowed down the cell proliferative rates in T24 cells (Figure 2B) and in 5637 cells (Figure 2C). In contrast, upregulation of Sema4D increased cell proliferation in SV-HUC-1 cells (Figure 2D). Furthermore, the colony formation was assayed in 2D (Figure 2E) and 3D (Figure 2H) cultures. In the 2D cultures, the Sema4D-depleted cells formed only about 150 colonies, resulting in nearly 70% decreases of colonies in both T24 cells and 5637 cells (Figure 2F). On the contrary, Sema4D-overexpressed T24 cells formed over 600 colonies, which significantly contrasted with the 360 colonies in control T24 cells (Figure 2G). In the 3D cultures, the

decrease of clonogenicity abilities by knockdown of Sema4D was up to 58.3% in 5637 cells (Figure 2I) and 66.7% in 5637 cells (Figure 2J), whereas the clonogenicity was increased by 120% in SV-HUC-1 cells after overexpression of Sema4D (Figure 2K). These observations suggested that Sema4D promoted cell viability in bladder cancer.

Sema4D controls G0/G1 transition to S phase during cell cycle in bladder cancer cells

The cell cycle was then analyzed in T24, 5637, and SV-HUC-1 cells under each treatment (Figure 3A). In T24 cells, as compared with control cells, the Sema4D-depleted

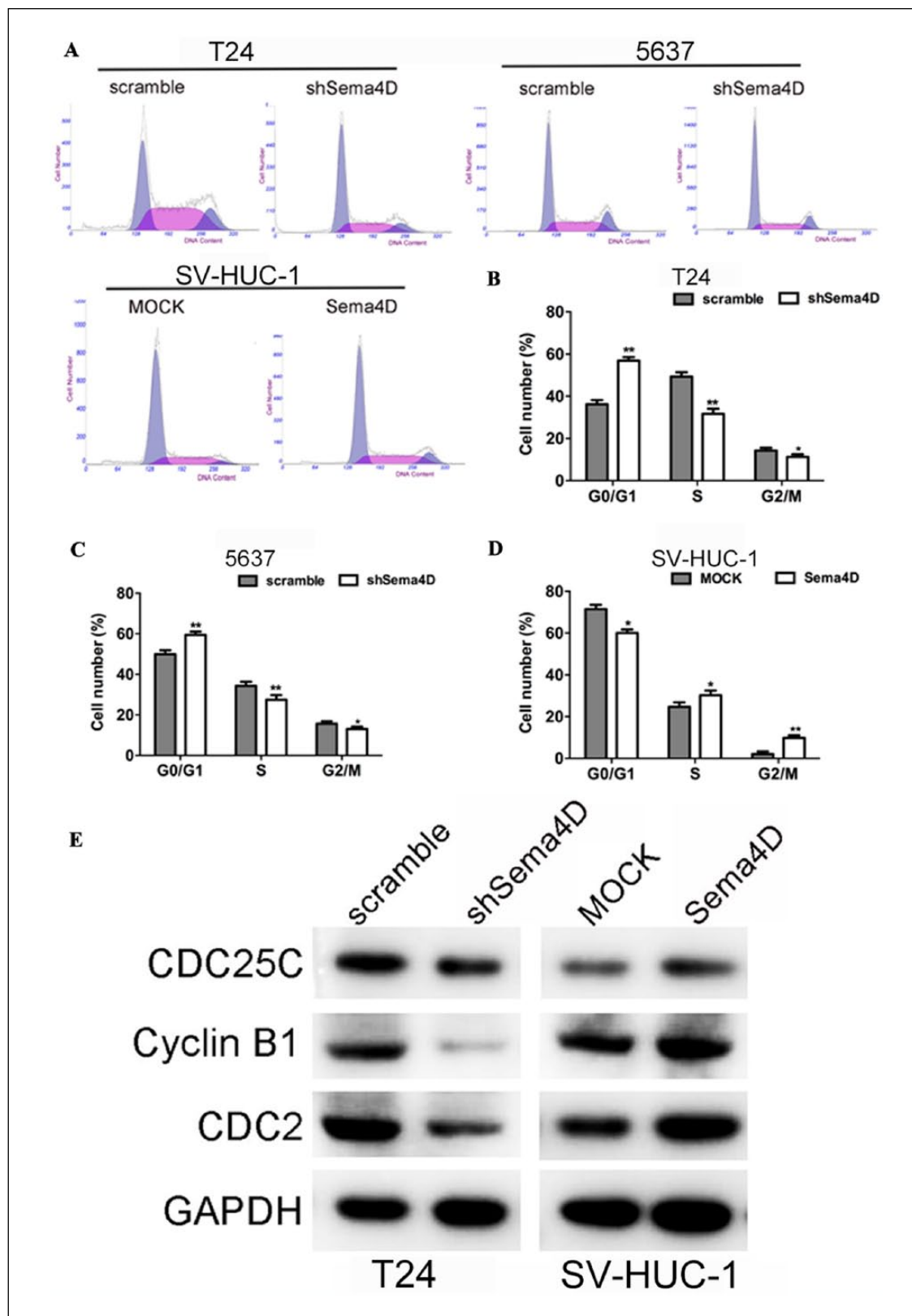


Figure 3. Sema4D is involved in cell cycle regulation in bladder cancer. (A) Cell cycle was analyzed in T24, 5637, and SV-HUC-1 cells with each treatment. (B–D) The cell proportion in G0/G1 phase, S phase, and G2/M phase were quantified in T24 cells, 5637 cells, and SV-HUC-1 cells with distinct treatments. (E) Western blot analysis of key cell cycle regulators CDC25C, Cyclin B1, and CDC2 in T24 cells and 5637 cells with treatments as indicated. * $p < .05$; ** $p < .01$.

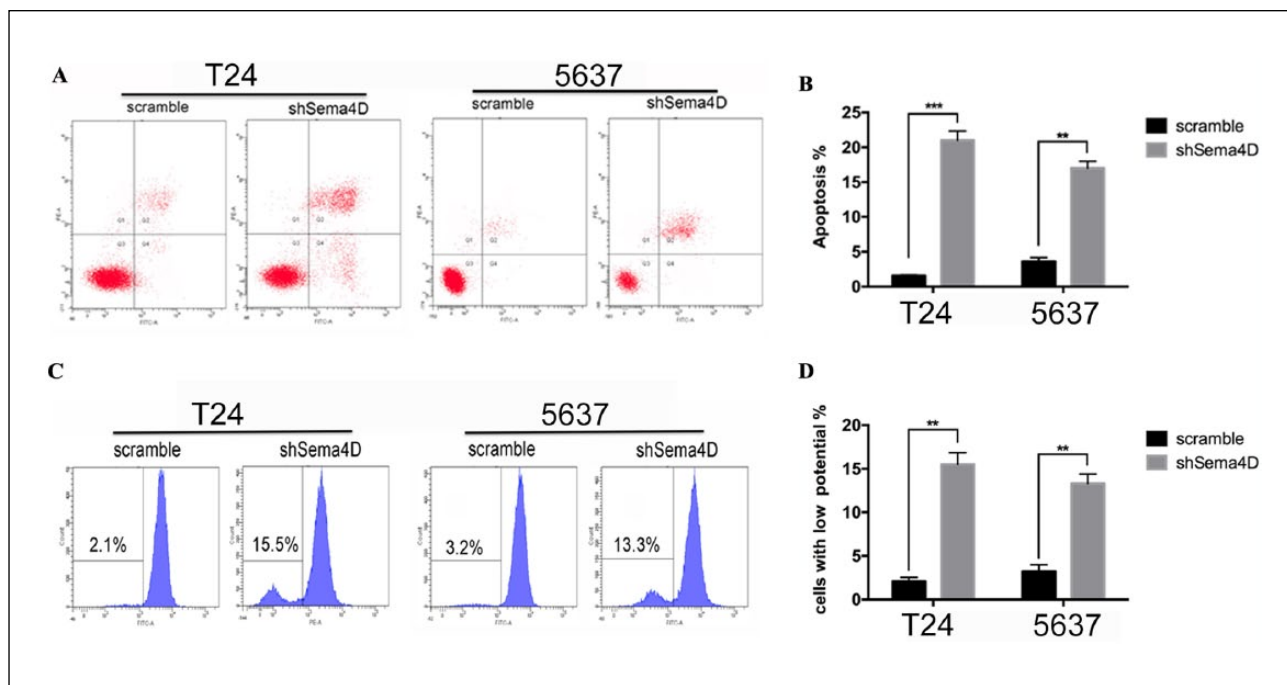


Figure 4. Knockdown of Sema4D induces cell apoptosis and increases the mitochondrial membrane potentials in T24 and 5637 cells. (A) Cell apoptosis was assessed using flow cytometry with annexin V-FITC/PI staining. (B) The apoptosis of T24 cells and 5637 cells with or without specific shSema4D transfection is shown. (C) Mitochondrial membrane potentials ($\Delta\Psi_m$) were determined after rhodamine 123 staining in T24 and 5637 cells after transfection with shSema4D. (D) In the $\Delta\Psi_m$ detection, low potentials were considered as apoptosis, while high potentials were survivals. The cells with low potentials were shown in T24 cells and 5637 cells. $**p < .01$; $***p < .001$.

cells had significantly higher cell proportion in the G0/G1 phase, while cell proportions in the S phase and the G2/M phase were decreased (Figure 3B). Comparable results were also observed in Sema4D-depleted 5637 cells (Figure 3C). In addition, when Sema4D was overexpressed in SV-HUC-1 cells, the cell proportion in the G0/G1 phase was decreased, with cell proportions in S phase and G2/M phase consistently increased (Figure 3D). Western blotting also revealed that knockdown of Sema4D in T24 cells decreased the expression of key cell cycle regulators such as CDC25C, Cyclin B1, and CDC2, and overexpression of Sema4D in SV-HUC-1 cells, on the contrary, increased the expression of key cell cycle regulators (Figure 3E). All these data suggested that Sema4D was involved in the cell cycle regulation in bladder cancer.

Knockdown of Sema4D induces cell apoptosis and increases the mitochondrial membrane potentials in T24 and 5637 cells

Next we assessed whether cell apoptosis and the mitochondrial membrane potential were affected by Sema4D. Since overexpression of Sema4D in SV-HUC-1 cells caused no significant changes in cell apoptosis (data not shown), cell apoptosis was assessed only in T24 cells and 5637 cells (Figure 4A). Interestingly, while the control

T24 cells were minimally apoptotic (~2%), as high as 20% of Sema4D-depleted T24 cells were apoptotic. The approximate 16% apoptosis rate in Sema4D-depleted 5637 cells also remarkably contrasted with the only 4% apoptosis of control 5637 cells (Figure 4B). Furthermore, the mitochondrial membrane potentials were determined in T24 and 5637 cells (Figure 4C). Cells with high $\Delta\Psi_m$ are marked as survival, and those with low $\Delta\Psi_m$ are considered as apoptosis. After knockdown of Sema4D, T24 and 5637 cells with low potentials were concordantly increased (Figure 4D). These data suggested that knockdown of Sema4D induced cell apoptosis via disrupting the mitochondrial membrane integrity.

Sema4D promotes cell metastasis in bladder cancer cell lines

We further assessed whether Sema4D exhibited any malignant behavior in bladder cancer cell metastasis. In the wound-healing assay, it was observed that knockdown of Sema4D inhibited wound recovery in T24 cells and 5637 cells, while overexpression of Sema4D promoted wound recovery in SV-HUC-1 cells (Figure 5A). The wound recovery rate was inhibited by 60% in T24 cells and 48% in 5637 cells after knockdown of Sema4D (Figure 5B), whereas it was increased by 40% after overexpression of

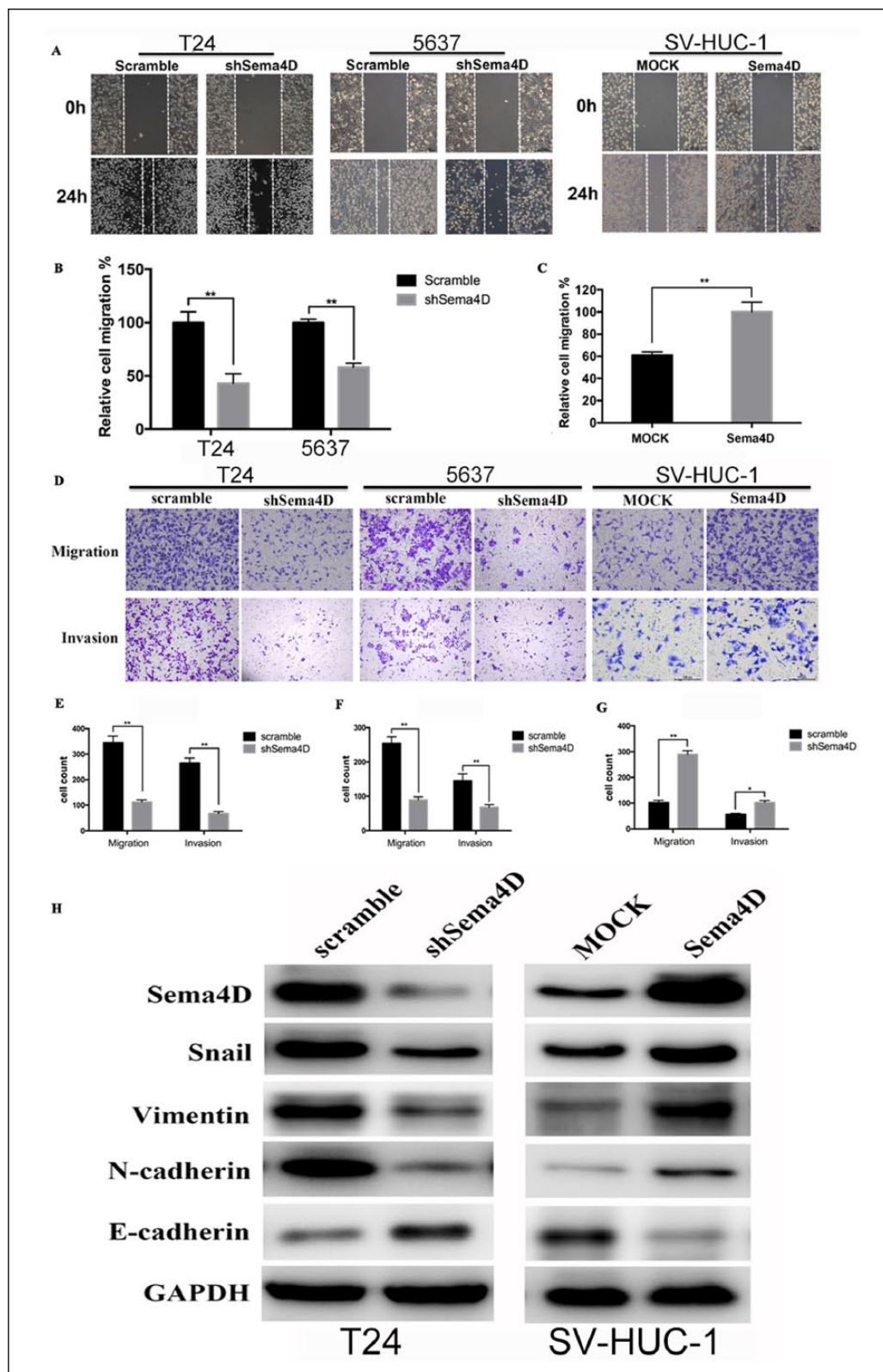


Figure 5. Sema4D promotes cell metastasis in bladder cancer cell lines. (A) Wound-healing assays were performed with T24, 5637, and SV-HUC-1 cells. shSema4D was transfected into T24 and 5637 cells, respectively, and Sema4D plasmid was transfected into SV-HUC-1 cells prior to tests. The representative images were taken after 24 hours of serum-free migration. (B, C) The relative wound recovery rates were calculated and are shown. (D) Transwell migration and invasion assays were performed. (E–G) The cells migrated and invaded to the lower surface of the chamber were counted in T24, 5637, and SV-HUC-1 cells. (H) Western blot analysis of the major epithelial mesenchymal transition biomarkers in Sema4D-depleted T24 cells and Sema4D-overexpressed SV-HUC-1 cells. * $p < .05$; ** $p < .01$.

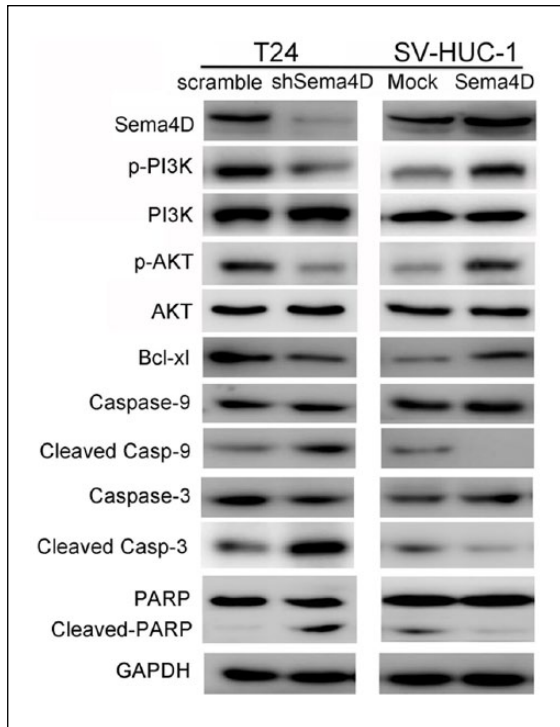


Figure 6. Sema4D activates the PI3K/AKT signal pathway in bladder cancer T24 and SV-HUC-1 cells. Western blot analysis was performed to detect the proapoptotic factors and PI3K/AKT signals in Sema4D-depleted T24 cells and in Sema4D-overexpressed SV-HUC-1 cells. p-PI3K: phosphorylated phosphatidylinositol3-kinase; p-AKT: phosphorylated AKT; PARP: poly-(ADP-ribose) polymerase.

Sema4D in SV-HUC-1 cells (Figure 5C). Transwell assays were further performed to verify the above phenomenon (Figure 5D). In the T24 cells, when Sema4D was depleted, migrated cells were only approximately 100, which contrasted with the over 350 migrated cells in the control group. This observation supported that knockdown of Sema4D inhibited cell migration. Moreover, the invasive T24 cells were also significantly decreased by up to 70% (Figure 5E). Similar results were also obtained in 5637 cells (Figure 5F). In turn, when Sema4D was overexpressed in SV-HUC-1 cells, both migrated and invaded SV-HUC-1 cells were significantly increased (Figure 5G). Furthermore, when Sema4D was depleted in T24 cells, the epithelial biomarker E-cadherin was upregulated, with mesenchymal biomarkers Snail, Vimentin, and N-cadherin decreased. In SV-HUC-1 cells, the above biomarkers were reversely expressed (Figure 5H). These data concordantly suggested that Sema4D promoted cell migration and invasion through epithelial mesenchymal transition (EMT).

Sema4D activates the PI3K/AKT signal pathway in bladder cancer cells

To explore the possible mechanisms underlying Sema4D biological activities in bladder cancer, T24 cells and

SV-HUC-1 cells were further subjected to Western blot analysis. Consistently, it was found that knockdown of Sema4D in T24 cells downregulated the expression of proapoptotic factors such as Bcl-x1, cleaved caspase-9, cleaved caspase-3, and cleaved PARP. Their expression was inversely observed after overexpression of Sema4D in SV-HUC-1 cells. More interestingly, we found that the PI3K as well as the phosphorylated AKT were increased after Sema4D overexpression in SV-HUC-1 cells and decreased after Sema4D depletion in T24 cells. The total protein levels of PI3K and AKT remained unchanged after neither Sema4D depletion nor overexpression (Figure 6). These data suggested that Sema4D activated the PI3K/AKT signal pathway in bladder cancer cell lines.

Sema4D promotes cell growth and metastasis in vivo

To further assess the effects of Sema4D on cell growth and metastasis in vivo, a mouse model bearing human bladder cancer using T24 cells or SV-HUC-1 cells were established. For T24 cell-established model, an equal amount of cells with Sema4D depleted or not were injected into mice. For SV-HUC-1 cell-established model, an equal amount of cells with Sema4D overexpression or not were injected into mice. Four weeks after inoculation, it was shown that the Sema4D-depleted group of mice exhibited remarkably smaller tumor sizes as compared with the control group. On the contrary, the group of mice with Sema4D overexpression exhibited significantly larger tumor sizes (Figure 7A). It was also observed that the Sema4D-depleted group of mice exhibited significantly smaller tumor volumes ever since the second week after inoculation and the difference in tumor volumes between 2 distinct groups was increasingly high as the time extended (Figure 7B). The significant differences in tumor volumes were also observed in Sema4D-overexpressed and control group of mice (Figure 7C). H&E staining verified the tumor cell morphology in each group. PCNA, a specific biomarker for proliferative cells, was intensely stained in Sema4D-overexpressed tumor tissues, while it was less stained in Sema4D-depleted tumor tissues relative to the corresponding control ones. Cleaved caspase-3, which is indicative of cell apoptosis, was strongly revealed after knockdown of Sema4D, but barely revealed after overexpression of Sema4D (Figure 7D). Furthermore, in the model of bladder cancer cell metastasis to lung, the number of mice that developed lung colonization was counted in both groups. The visual colonization was confirmed by H&E staining (Figure 7E). It was shown that only 1 out of the 10 mice in the shSema4D-depleted group exhibited lung colonization, while 6 out of 10 in the control group exhibited lung colonization (Figure 7F). These data suggested that knockdown of Sema4D inhibited cell metastasis in vivo. In other words, Sema4D promoted tumor growth and metastasis in a mouse model of bladder cancer.

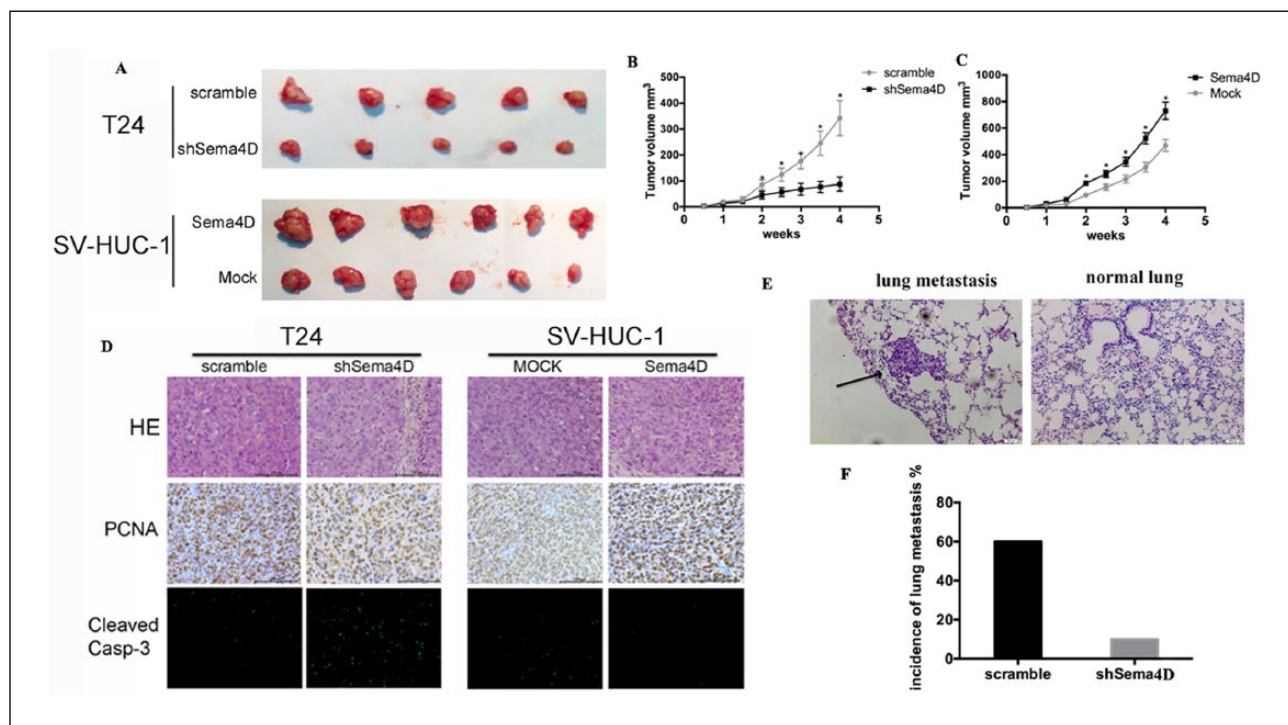


Figure 7. Sema4D promotes cell growth and metastasis in vivo in a xenotransplanted bladder cancer model. A mouse model bearing human bladder cancer using T24 cells or SV-HUC-1 cells was established. (A) The tumors dissected from each group of mice are shown. (B, C) Tumor volumes originating from T24 cells and SV-HUC-1 cells were measured for 4 consecutive weeks. (D) Hematoxylin & eosin (HE) staining, immunohistochemistry staining of the proliferating cell nuclear antigen (PCNA), and immunofluorescence analysis of cleaved caspase-3 were performed with the bladder cancer tissues from the mouse model. (E, F) Colonization in lung was observed and counted in the control and Sema4D-depleted group of mice. * $p < .05$.

Discussion

The progression of bladder cancer is attributed to the accumulation of gene mutations and/or aberrant gene expression. Critical genes that have been reported to be either mutated or aberrantly expressed include p53 and epigallocatechin-3-gallate (*EGCG*).¹⁰⁻¹² However, it is difficult to address all the clinicopathologic features of bladder cancer, and the dismal prognosis necessitates the search for a novel molecule that play a critical role in the progression of bladder cancer.

Sema4D is a member of the semaphorin family that is phylogenetically conserved and structurally enriched in cysteine-rich sema domain.² Sema4D expression in malignant solid tumors is significantly higher and closely related to the prognosis of patients. In cervical cancer, the high expression of Sema4D indicated that the overall survival time of patients was significantly shortened, which may be related to Sema4D mediating tumor angiogenesis, thus promoting the distant metastasis of cancer cells.¹³ In addition, studies in ovarian cancer and osteosarcoma have found that increased expression of Sema4D predicts progression-free survival and overall survival for patients without disease, which was shorter than that of the control group.⁷ One of the important functions of Sema4D was the

induction of tumor neovascularization. As an important angiogenic factor, Sema4D could induce the migration of endothelial cells and the formation of lumen.¹⁰ Therefore, Sema4D can become an antitumor angiogenesis target.

In breast cancer, Sierra et al.¹⁴ found that Plexin-B1 and c-met formed a complex on the surface of breast cancer cells, and Sema4D binds to this complex to activate tyrosine kinase activity of c-met. C-met activation was required for Sema4D-induced tumor angiogenesis. C-met signaling was a condition for cell migration and invasion growth mediated by Sema4D and Plexin-B1.¹⁵ In addition, Sema4D also plays an important role in bone metastasis of breast cancer. The bone metastasis of breast cancer does not directly destroy the bone, and it mainly inhibits the differentiation and maturation of the osteogenic precursor cells through a series of regulatory mechanisms.¹⁶ Wannemacher et al.¹⁷ knocked down the mice Sema4D gene, and the results exhibited obvious osteosclerosis characteristics, suggesting that Sema4D played an important role in osteogenesis and osteoclast balance. This suggested that Sema4D may also be involved in osteolytic destruction and inhibition of osteogenic differentiation.

Kato et al.¹⁸ reported that binding of Sema4D to Plexin-B1 activated the small molecule Ras homologue gene family A-Ras, resulting in the phosphorylation of

PYK2 and Src, thereby enhancing invasive ability of pancreatic cancer cells. The expression of Sema4D was related to the stage of pancreatic cancer, lymph node metastasis, tumor endothelial cell migration, and tumor angiogenesis.¹⁹ Tumor-derived Sema4D promoted the generation of tumor blood vessels, and after the formation, the blood vessels could further recruit endothelial cells.⁸ Endothelial cells stabilized and protected neoplastic blood vessels and induced the production of ANGPTL4, promoting angiogenesis, vascular permeability, and tumor metastasis.²⁰

The present study investigated the role of Sema4D in cell proliferation and metastasis in bladder cancer. The expression of Sema4D was initially confirmed to be upregulated in bladder cancer tissues. Since Sema4D was highly expressed in T24 cells and 5637 cells, shSema4D was used to deplete Sema4D expression, whereas the lowest expression of Sema4D in SV-HUC-1 cells made us adopt an expression plasmid to upregulate the expression of Sema4D in SV-HUC-1 cells. Subsequently, knockdown of Sema4D inhibited while overexpression of Sema4D promoted cell proliferation in the MTT assay. This effect was further confirmed in an anchor-independent colony formation assay. Cell migration and invasion were also controlled by Sema4D, as evidenced by the observations that Sema4D depletion suppressed the wound recovery process, migration, and invasion abilities, and Sema4D overexpression reversed the above inhibitive effects as well as induced the EMT process. The Sema4D-mediated tumor growth and metastasis was also strengthened in a mouse model bearing human bladder cancer. Sema4D-promoted cell metastasis was also confirmed in a mouse model of bladder cancer cell metastasis to lung. In all, the present study identified Sema4D as a key mediator of cell proliferation and metastasis in bladder cancer in vitro and in vivo.

One interesting finding was the activation of PI3K/AKT pathway by Sema4D. Previous evidence has suggested that PI3K/AKT signaling plays an essential role in numerous pathophysiologic events including tumorigenesis.²¹ PI3K triggers a signal cascade that generates phosphatidylinositol triphosphate (PIP3) and activates AKT. Activated AKT functionally regulates cell growth and survival.²² Our study identified Sema4D as a critical mediator of cell proliferation and metastasis in bladder cancer. The malignant behaviors of Sema4D in bladder cancer were achieved by activating the PI3K/AKT pathway. Moreover, activation of the PI3K/AKT signaling pathway is closely related to tumor angiogenesis in bladder cancer. Therefore, the role of Sema4D in angiogenesis should be further investigated in bladder cancer to determine whether Sema4D can induce tumor angiogenesis and promote bladder cancer metastasis, as reported in breast cancer.

Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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