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Propofol Inhibits the Proliferation, Migration, and Stem-like Properties of Bladder Cancer Mainly by Suppressing the Hedgehog Pathway

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Abstract

Bladder cancer is one of the most common malignancies. The existence of bladder cancer stem cells (BCSCs) has been suggested to underlie bladder tumor initiation and recurrence. Propofol is a commonly used intravenous anesthetic. Here, we find that propofol can dramatically block the activation of Hedgehog pathway in BCSCs. The propofol strongly repressed the growth of cancer cells. Attenuated proliferation and enhanced apoptosis of tumor cells were observed upon propofol stimulation. Furthermore, propofol reduced the self-renewal ability of BCSCs as well as the tumor formation. In conclusion, propofol is potentially used as a novel therapeutic agent for bladder cancer by targeting self-renewal through inhibiting Hedgehog pathway.

Keywords

bladder cancer, propofol, self-renewal, BCSCs

Introduction

Bladder cancer is the ninth most common malignant tumor in the world and is a highly heterogeneous disease[1,2]. Surgical excision is the main method for the treatment of solid tumors, but recurrence and metastasis are easy to occur after surgery and affect the prognosis of patients. The effects of various factors such as invasive operation and drugs used during perioperative period on tumors have attracted extensive attention. One important factor is the use of anesthetic drugs[3].

Propofol is a commonly used intravenous anesthetic. It is characterized by rapid effect, short-term sedation, and less adverse reactions, which is widely used in general anesthesia for tumor resection surgery. Numerous literatures have reported that propofol not only has anesthetic effect, but also has immunomodulatory effects, especially for anti-inflammatory property. In addition, the roles of propofol in biological behavior of tumors are controversial[4,5]. Mounting evidence has stated propofol inhibits the invasion and metastasis of various tumor cells such as lung cancer, ovarian cancer, esophageal cancer, and breast cancer to different degrees[6,7]. Conversely, cell proliferation and migration of breast cancer cells are promoted by propofol via downregulation of p53 and activation of Nrf2 pathway[8]. At present, the effect and mechanism of propofol in bladder cancer are not clear, and precise investigation is of importance.

Cancer stem cells (CSCs) have the potential of self-renewal and multidirectional differentiation, which they are considered to play a key role in the occurrence and development of tumors, although few of them exist in tumors[9–11].

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Bladder cancer stem cells (BCSCs) are tumor cells with multidirectional differentiation potential, which are uniquely capable of initiating and sustaining tumor growth[12]. Its resistance to chemotherapy drugs caused the bladder cancer easy to evade death, survive therapeutic intervention, and result in recurrence and metastasis[6]. BCSCs can be used as ideal targets for cancer drugs and can control the occurrence and development of tumor from the source[13,14]. In this study, cytological experiments and model of bladder cancer in nude mice were made to observe the effect of propofol on the growth of xenograft tumor in nude mice, and to explore the effect of propofol on the self-renewal of BCSCs and elucidate its possible mechanism.

Materials and Methods

Cell Culture and Treatments

Bladder cancer T24 cells were from Institute of Biophysics, Chinese Academy of Sciences (Beijing, China). Cell lines 5637 and SW780 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA, USA) in a humidified incubator at 37°C with a gas mixture of 5% CO_2 and 95% air. When monolayer cultures reached confluency, cells were subcultured using the Trypsin (Life Technologies, Carlsbad, CA, USA).

For stimulation of propofol, cells were incubated in culture medium containing diverse doses of propofol (0 to 8 μg/ml, Sigma-Aldrich, Shanghai, China) for 48 h. Dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) was used in these experiments as well as the naïve control. Before treatment, bladder cancer cells were cultured at the density of 1×10^6 per ml on a 30-mm^3 Petri dishes, as described above. Propofol was diluted with cell medium to the desired concentrations.

Animal Experiments

Nude mice were obtained from the Animal Center of the Chinese Academy of Medical Science (Beijing, China). Female 6-week-old nude mice with a body weight of approximately 15 g were used and kept under specific pathogen-free conditions. Xenografts of T24/5637 and SW780 cells were produced by injecting tumor cells (1×10^6 resuspended in phosphate buffered saline) subcutaneously into the back of mice. When tumors reached a diameter of 3 to 5 mm, the mice were grouped (12 mice/group) and administered 4 μg/ml propofol or DMSO intraperitoneally, three times per week for 30 days. Tumor size was measured twice per week.

[^{3}H]-TdR Incorporation Assay

Bladder cancer cells were seeded on 96-well culture plates, cultured until the cells reached 70% to 80% confluency, serum starved in DMEM for 24 h, stimulated with propofol or DMSO for 72 h, pulsed with [^{3}H]-thymidine for 4 h, and then their [^{3}H]-thymidine incorporation was measured in the liquid scintillation counter LKB1219.

Cytotoxicity Assay

Cytotoxicity assay was performed as described previously[15]. Propofol or DMSO control at the indicated final concentration was added to the 51Cr-labeled target cells immediately before adding effector cells. Each assay was set up in triplicate, and the results were expressed as the percentage of specific lysis.

IC50 Determination

Bladder cancer cells from patients were seeded in 96-well plates and treated with various concentrations of propofol dissolved in 10% FBS medium for 24 h. IC50 was calculated using Cell Counting Kit-8 (Sigma, Darmstadt, Germany).

Real-time Polymerase Chain Reaction

Total RNA from cell lines was extracted with an RNA isolation kit (Tiangen Biotech, Beijing, China). RNA was subjected to cDNA synthesis with a PrimeScript RT reagent kit (Takara Bio, Shiga, Japan). cDNA was used as the template for real-time polymerase chain reaction (PCR) analysis on an ABI 7200 analyzer (Applied Biosystems, Waltham, MA, USA) with the fluorescent probe SYBR Green I (Tiangen Biotech, Beijing, China). Relative expression levels of the genes were normalized to the housekeeping gene GAPDH. Each experiment was independently repeated at least three times.

Apoptosis Assay

Propofol or DMSO-treated bladder cancer cells were stained with an Annexin V-fluorescein isothiocyanate (FITC) kit (Sigma-Aldrich, Gilingham, UK). Cells were washed, digested, collected, resuspended, stained with annexin V-FITC and propidium iodide (BioVision, Milpitas, CA, USA), and incubated for 10 min at room temperature in the dark. Annexin V-positive cells were analyzed with a FACS-Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Cell Attachment Assay

Cell substrate attachment assays were performed using a modification of the method described. Briefly, 96-well plates were incubated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and blocked with heat-denatured bovine serum albumin (Sigma, St. Louis, MO, USA) for 1 h. Near-confluent cells were harvested with trypsin and resuspended in DMEM with 1% FBS, and recovered at 37°C for 15 min. Attached cells were fixed and stained with crystal

violet (Sigma, St. Louis, MO, USA). The absorbance of each well was measured at 575 nm with an ELISA reader.

Cell Migration Assay

A six-well plate with markings on the outer bottom of the plate was used as reference points during image acquisition, and cells were seeded per well. When the cell confluence reached 90% or more, a wound was made by scraping the cell monolayer along the previously drawn lines with a 200 μl pipette tip. The plate was rinsed three times to remove the cell debris, followed by adding DMEM that containing 4 μg/ml propofol with 10% FBS. Images were captured by a phase contrast microscope at 0 and 24 h of culture. The cell migration distance was calculated as follows: migration distance $=$ distance$_{24\ h}$ $-$ distance$_{0\ h}$. The cell migration rate was calculated according to the formula: (migration distance$_{propofol}$/migration distance$_{control}$) $\times$ 100%

Colony Formation Assay

Bladder cancer cells were suspended with soft agar culture media into 24-well plates at a density of 1,000 cells/well. After 2–3 weeks, colonies ($\geq$10 cells) were counted and photographed.

Sphere-formation Assay

Single bladder cancer cells were seeded into six-well plates with ultralow attachment surfaces (Corning, NY, USA). Cells were cultured in DMEM media. Sphere number was calculated 2 weeks later.

5-Bromo-20-deoxyuridine and Ki-67 Analyses

5-Bromo-20-deoxyuridine (BrdU, Roche, Basel, Switzerland) was added into nonadherent spheres. After 4 h, cells were fixed, permeabilized, DNase treated, and stained with anti-BrdU antibody (BD Pharmingen, San Jose, USA). For Ki-67 (51-36525X, BD Pharmingen) analyses, cells were fixed and permeabilized before the intracellular stain. Cells were analyzed using the BD LSR II or FACSCanto II flow cytometers.

Pathway Reporter Array

Profile of the changes in the activities of 45 signaling pathways in propofol and DMSO-treated cells was evaluated using the Cignal 45-Pathway Reporter Array (QIAGEN, Manchester, UK). Dual-luciferase reporter assays, with a pathway-specific firefly luciferase reporter and a constitutively expressed Renilla reporter serving as a normalization control, were performed.

Ethics

Informed consent was obtained from all patients and animal experiments were conducted in accordance with the Institutional Animal Care Guidelines of Peking University Third Hospital, China. This study was approved by Peking University Third Hospital Medical Science Research Ethics Committee, Beijing, China.

Statistical Analysis

All data were analyzed using SPSS 24.0. Kaplan–Meier analysis was used to estimate cumulative cause-specific survival rates in survival between mice administered propofol or DMSO. The differences between two groups were analyzed by unpaired Student's *t*-test. The differences among three or more groups were analyzed using one-way analysis of variance, followed by post hoc test. All of the data were obtained from three separate experiments and expressed as mean $\pm$ standard deviation, and the statistical difference was determined as $^{*}P < 0.05$ or $^{**}P < 0.01$.

Results

Propofol Suppressed the Proliferation and Self-renewal Ability of Tumor Cells

The plasma concentration range of propofol is 3 to 6 μg/ml during clinical surgery. In order to distinguish the effects of propofol on cancer cell proliferation, experiments were performed with different concentrations 0, 2, 4, and 8 μg/ml. Propofol exposure induced a marked decrease in proliferation in 4 μg/ml, and not obvious in 8 μg/ml (data not shown). So we treated bladder cancer cells with 4 μg/ml in this study[16,17], which is also commonly used in clinical.

When propofol was added into the culture medium, the [^{3}H]-thymidine incorporation of T24, 5637, and SW780 in propofol-treated group was 6127 $\pm$ 637, 5879 $\pm$ 579, and 5679 $\pm$ 537, which was significantly lower than 8053 $\pm$ 735, 7876 $\pm$ 703, and 7728 $\pm$ 768 in the DMSO-treated group at 72 h, indicating the growth of bladder cancer cells was all repressed (Fig. 1A). CSCs are the primary cells that initiate tumor formation. The self-renewal ability of bladder cancer cells with propofol was investigated. When tumor cells were put into the dish coated with Matrigel, propofol reduced the attachment of cancer cells to the matrix. To be specific, the attachment percentage of T24, 5637, and SW780 in propofol-treated group was 37.2 $\pm$ 2.8%, 47.6 $\pm$ 4.2%, and 58.3 $\pm$ 3.9%, compared with normalized DMSO-treated group (Fig. 1B). Meanwhile, the migration percentage of T24, 5637, and SW780 in propofol-treated group was 47.1 $\pm$ 3.7%, 37.2 $\pm$ 1.3%, and 42.6 $\pm$ 3.5%, compared with normalized DMSO-treated group, so the migration of the tumor cells was attenuated by propofol (Fig. 1C). In colony formation assay, propofol decreased the colony number of tumor cells. To be specific, the colony percentage of T24, 5637,

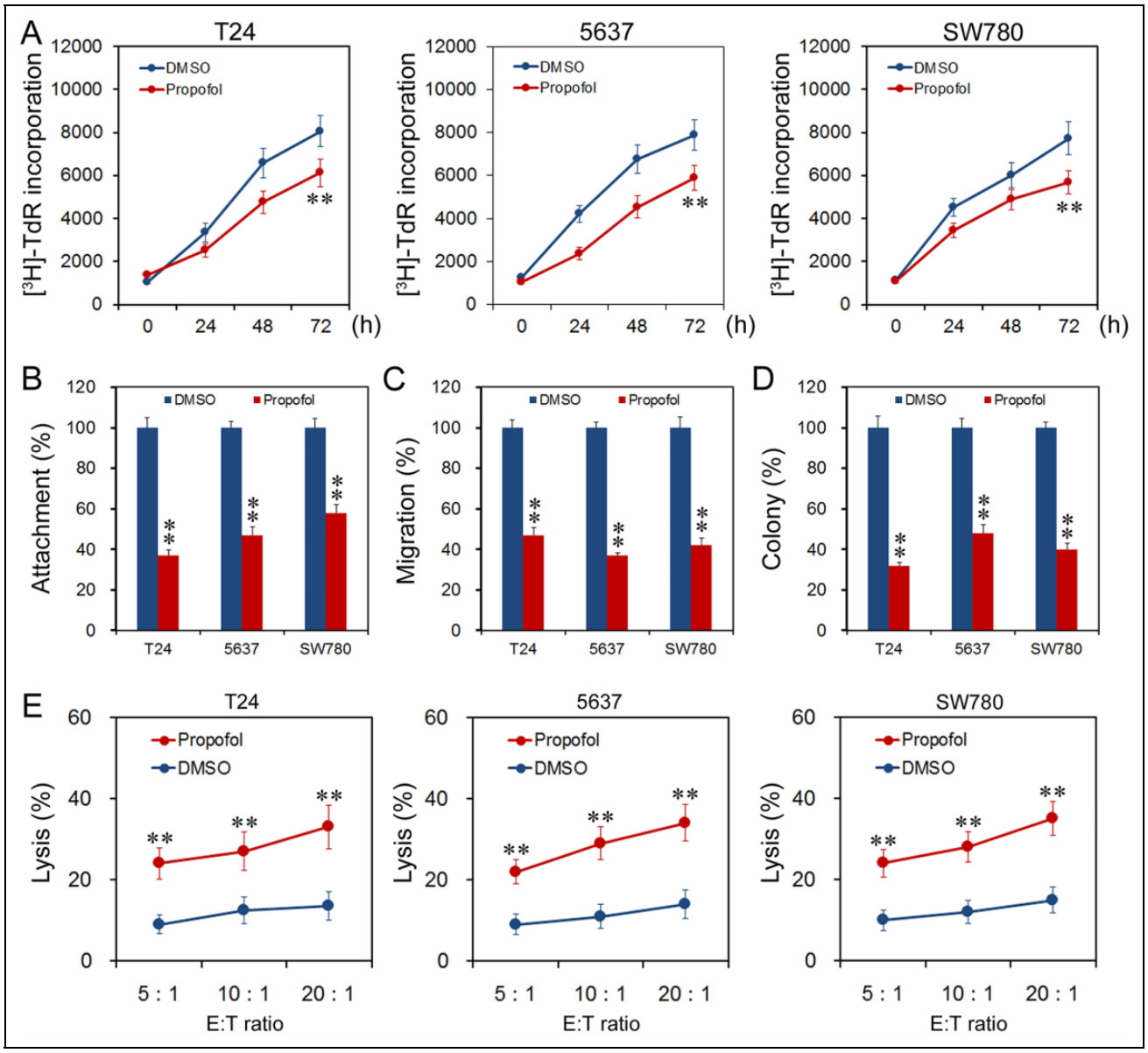

Figure 1. Propofol suppressed the proliferation of tumor cells. (A) Inhibition of the bladder cancer cell growth *in vitro*. Bladder cancer cells were incubated by DMSO and propofol for indicated time. The incorporation of [3H]-TdR into tumor cells was examined. (B) Reduction in tumor cell attachment to Matrigel by propofol. (C). Tumor cell migration through transwell was suppressed by propofol. (D) Inhibition of tumor cell colony formation by propofol. (E) Propofol exhibited the lysis of human bladder cancer cells by NK cells. DMSO: dimethyl sulfoxide; NK: natural killer.

and SW780 in propofol-treated group was 32.2 ± 1.6%, 48.3 ± 4.3%, and 40.5 ± 2.9%, compared with normalized DMSO-treated group (Fig. 1D). The microscopic figures of cell migration and clone formation were presented in supplemental material. In addition, propofol also exhibited the lysis of human bladder cancer cells by natural killer cells, and the lysis percentage of T24, 5637, and SW780 in propofol-treated group was 33.6 ± 5.3%, 34.2 ± 4.5%, and 35.1 ± 4.2%, which was significantly higher than 13.5 ± 3.5%, 14.1 ± 3.6%, and 15.2 ± 3.2% in DMSO-treated group (Fig. 1E).

Propofol Inhibited Self-renewal Ability of Tumor Cells

In propofol-treated group, the CD44 expression of bladder cancer cells T24, 5637, and SW780 was 35.2 ± 2.3%, 30.3 ± 4.1%, and 46.2 ± 3.5%, respectively, which was significantly lower than that of in DMSO-treated group (Fig. 2A), indicating propofol downregulated CD44 expression in bladder cancer cells. Meanwhile, the oncosphere numbers of bladder cancer cells T24, 5637, and SW780 was 39.3 ± 5.2%, 48.6 ± 4.3%, and 33.7 ± 2.7%, respectively, which was significantly lower than that of in

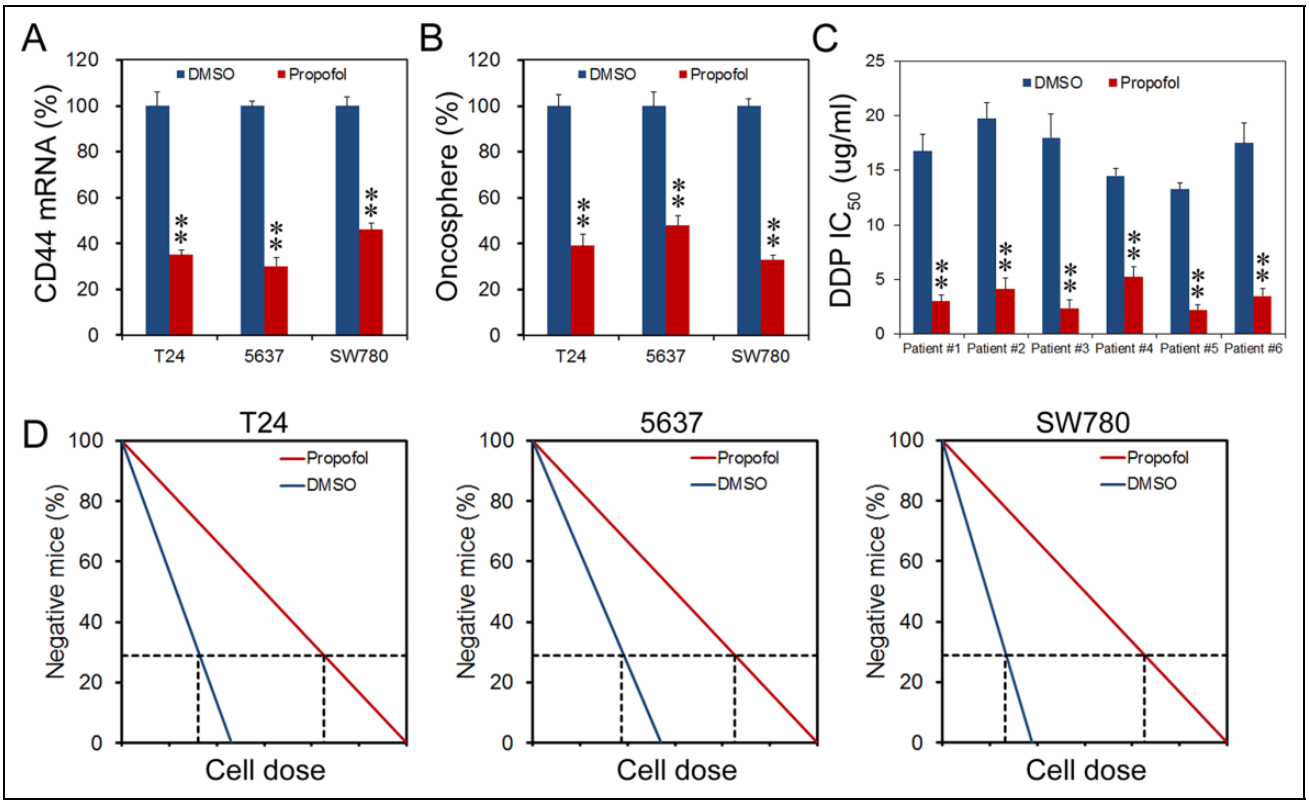

Figure 2. Propofol inhibited self-renewal ability of tumor cells. (A) Real-time PCR measurement of relative CD44 mRNA expression in vehicle-treated tumor cells and in tumor cells treated with propofol. (B) Oncosphere numbers were decreased by propofol. (C) The cisplatin (DDP) IC50 of tumor cells was significantly decreased by propofol incubation. (D) Tumor formation capability was attenuated by propofol. PCR: polymerase chain reaction.

DMSO-treated group, indicating that propofol reduced the oncosphere numbers of bladder cancer cells (Fig. 2B), and the microscopic figures of sphere formation were showed in supplemental material. CSCs are resistant to many drugs, so we determined the IC50 of cisplatin (DDP) on bladder cancer cells from six patients. In DMSO-treated group, the IC50 was 16.5 ± 1.5, 19.7 ± 1.4, 18.2 ± 2.2, 14.5 ± 0.7, 13.2 ± 0.6, and 17.6 ± 1.8 μg/ml, but propofol-treated cancer cells had a significantly lower IC50: 3.2 ± 0.6, 4.1 ± 1.0, 2.5 ± 0.8, 5.3 ± 0.9, 2.5 ± 0.4, and 3.5 ± 0.6 μg/ml, indicating that propofol can promote DDP sensitivity (Fig. 2C). Finally, the tumor formation by bladder cancer cells with serial dilution in mice was examined. The propofol-treated cells needed a higher cell number to form tumor in 30% mice when compared with the control (Fig. 2D).

Propofol Suppressed the Proliferation and Enhanced the Apoptosis of Tumor Cells

The S phase of bladder cancer cells T24 and 5637 in propofol-treated group was decreased 0.57 ± 0.08 and 0.67 ± 0.05 fold compared with DMSO-treated group, but the G0 phase of two bladder cancer cells in propofol-treated group was increased 1.21 ± 0.08 and 1.18 ± 0.12 fold compared with DMSO-treated group (Fig. 3A, B), indicating the proliferation of tumor cells could be attenuated by propofol. Meanwhile, the cell apoptosis of cancer cells was increased by propofol in a dose-dependent manner (Fig. 3C).

The Underlying Mechanism of Tumor Suppressing Function of Propofol

To further identify the underlying mechanism of tumor suppressing function of propofol in bladder cancer, the activities of a total of 45 signal transduction pathways were compared between propofol-treated and control groups. We displayed nine pathways that are the most related to the stemness in BSCSs. The results showed that Hedgehog pathway was the leading pathway that was significantly inhibited in propofol-treated cells (about 4.5-fold downregulation VS DMSO-treated group; Fig. 4A). To validate the results of pathway reporter array, we performed real-time PCR and found that propofol treatment induced significantly lower Gli1/Gli2/Jag2 mRNA expression in tumor cells. Compared with normalized DMSO-treated group, the Gli1 mRNA level of T24,

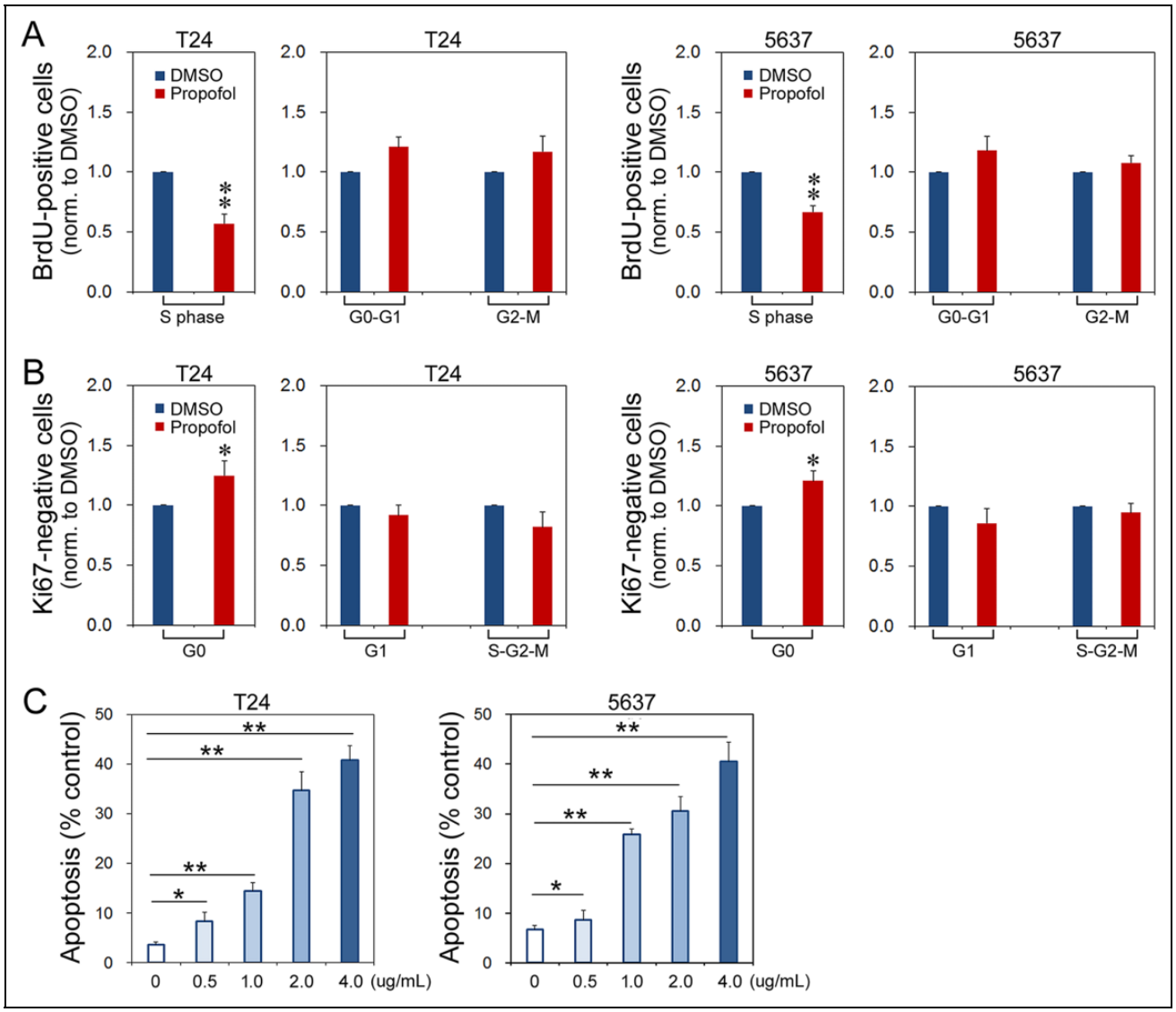

Figure 3. Propofol suppressed the proliferation and enhanced the apoptosis of tumor cells. Tumor cells T24 and 5637 were treated with propofol, the cell cycle was determined with BrdU staining (A) and Ki-67 staining (B). (C) Tumor cells were treated with propofol at indicated concentrations and the cell apoptosis was determined. BrdU: 5-bromo-20-deoxyuridine.

5637, and SW780 in propofol-treated group was 36.1 ± 2.9%, 42.3 ± 4.8%, and 32.1 ± 1.9%; the Gli2 mRNA level of T24, 5637 and SW780 in propofol-treated group was 36.1 ± 4.9%, 42.3 ± 2.3% and 32.1 ± 3.6%; and the Jag2 mRNA level of T24, 5637, and SW780 in propofol-treated group was 27.3 ± 1.6%, 35.6 ± 3.3%, and 43.2 ± 4.2%(Fig. 4B–D), indicating that the inhibition of tumor growth by propofol could be attributed to the inhibition of Gli1/Gli2/Jag2 activity.

Propofol Decreased the Tumor Formation of Bladder Cancer Cells

Next, we evaluated the therapeutic effect of propofol against bladder cancer cells in mice xenografts. We transplanted three bladder cancer cell lines (T24, 5637, and SW780) into 12 nude mice, respectively, and then treated them with either propofol or DMSO. The tumor volume of three bladder cancer cell lines (T24, 5637, and SW780) in propofol-treated group was 592 ± 67, 683 ± 90, 613 ± 81 mm^3 at 30 days, which was significantly smaller than that of in DMSO-treated group (Fig. 5A). Meanwhile, the tumor weight of three bladder cancer cell lines (T24, 5637, and SW780) in propofol-treated group was 203 ± 72, 172 ± 83, and 195 ± 92 mg at 30 days, which was significantly lighter than that of in DMSO-treated group (Fig. 5B). The results indicated that propofol significantly reduced the tumor volume and tumor weight. Moreover, propofol enhanced the survival time (Fig. 5C) of mice, indicating the potential for propofol as a therapy against bladder cancer.

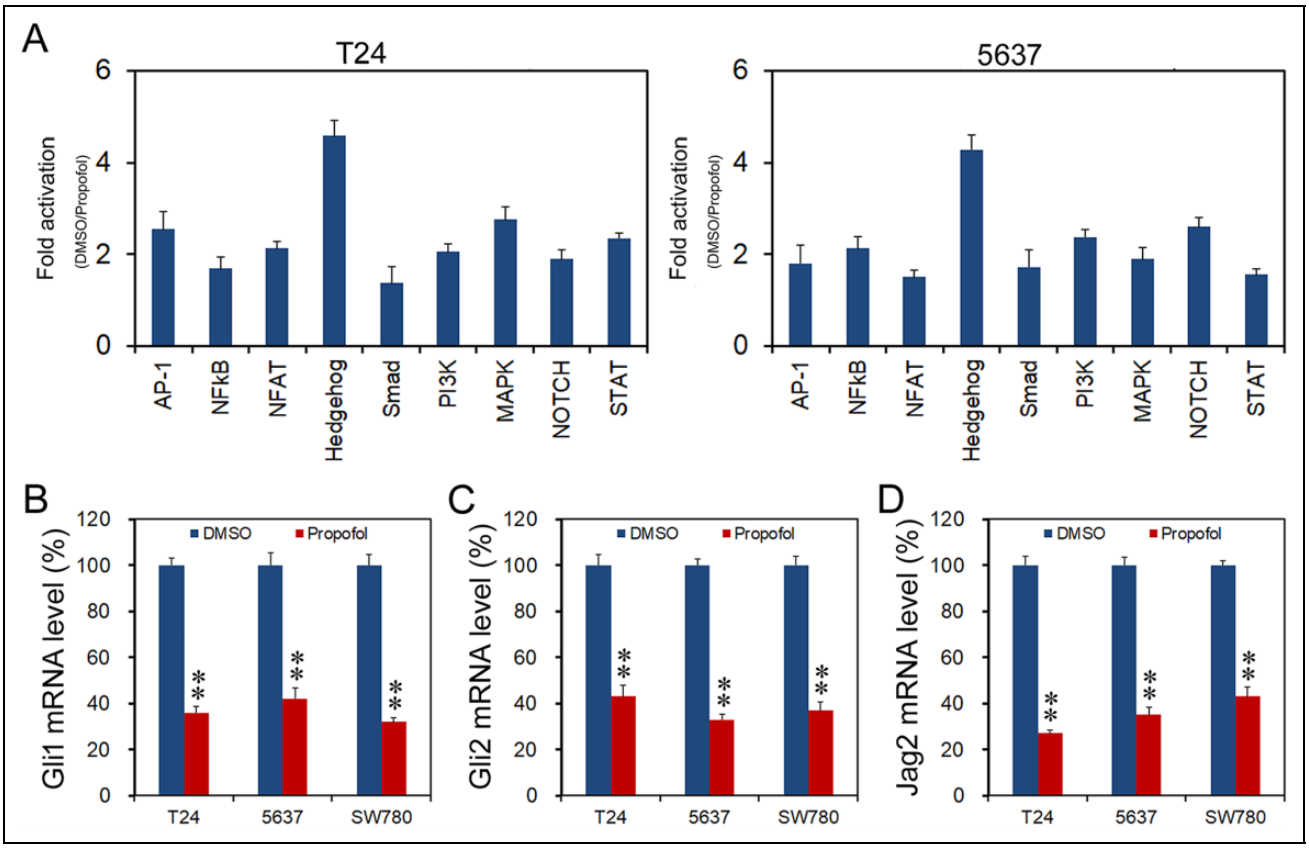

Figure 4. Propofol inhibits tumorigenesis via Hedgehog signaling in bladder cancer. (A) Pathway array analysis of gene expression from DMSO and propofol-treated tumor cells. (B–D) Real-time PCR measurement of relative Gli1/Gli2/Jag2 mRNA expression in tumor cells with propofol or DMSO. DMSO: dimethyl sulfoxide; PCR: polymerase chain reaction.

Discussion

Propofol is a commonly used anesthetic drug in clinical settings, which has good anesthetic induction and maintenance effects, and is often used in radical surgery of tumors and postoperative sedation[18]. In addition to anesthesia, propofol also affects immunity, inflammation, and ischemia reperfusion injury[4,19]. Propofol has been reported to induce antitumor immunity by promoting T-assisted cell activation and differentiation[20–22] and can inhibit the viability, migration, and invasion of bladder cancer T24 cells *in vitro*[23]. In this study, we explored the effects of propofol on the proliferation, migration, and stem-like properties in three bladder cancer cell lines, identified its underlying mechanisms, and further validated its effects in primary human bladder cancer cells and mice xenografts *in vivo*.

BCSCs are tumor cells with multiple differentiation potential in bladder cancer tissues, which are considered as the main factors of bladder cancer initiation and recurrence, and whose stemness is regulated by genetics and epigenetics[24–26]. Molecules and signaling pathways in BSCSs play an important role in regulating their proliferation and differentiation[27]. The use of chemotherapy drugs can significantly reduce the volume of bladder cancer and promote the apoptosis of tumor cells, but on the other hand, it promotes the growth of BCSCs[28,29]. Targeted treatment of BCSCs is important for the treatment of bladder cancer. Understanding the effects of perioperative anesthetics on CSCs is helpful in optimizing the selection of anesthetics during the perioperative period, and researching and discovering targeted drugs for the treatment of bladder cancer.

Our study suggests that propofol can inhibit the proliferation of bladder cancer cells and promote their apoptosis. The invasion, migration, and cloning ability of bladder cancer cells were inhibited. Further studies have shown that propofol can interfere with the DNA synthesis phase of bladder cancer cells, reduce the synthesis of Ki-67 protein, and thus inhibit the proliferation of tumor cells. The characteristics of CSCs are also expressed in the ability to form a ball and dilute into tumor. Propofol inhibits the formation of new tumors from a smaller number of bladder cancer cells and enhances the ability of CSCs to self-renew. CD44 is considered a marker of BCSCs[30,31]. Tatokoro et al.[32] demonstrated that the CD44+ cell subset of bladder transitional cell carcinoma cell line 5637 has the characteristics of bladder cancer

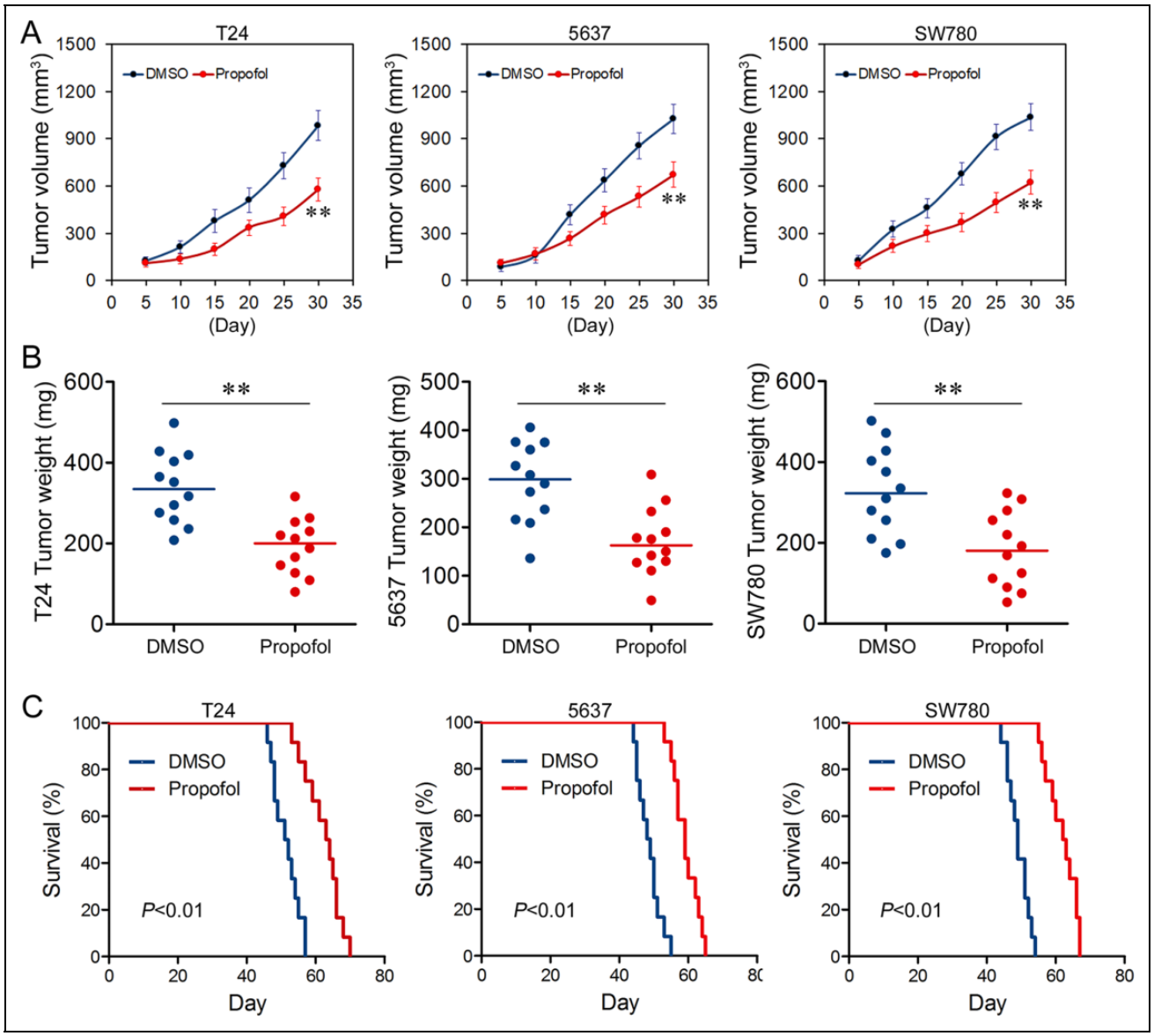

Figure 5. Propofol decreased the tumor formation of bladder cancer cells. Tumor growth (A) and weight (B) were reduced by propofol *in vivo*. Tumor cells were inoculated to nude mice ($n = 12$). Tumor growth was monitored and tumor weight was determined at day 30. (C) Survival of mice was increased by propofol treatment.

promoter cells and is more resistant to DDP. The chemoresistance of CSCs promotes tumor progression and distant metastasis. Our study suggests that propofol can reduce the expression of CD44 compared with the control group, indicating inhibition of tumor cell stemness. The use of propofol can promote the killing ability of the commonly used chemotherapy drug DDP on tumor cells, which may help inhibit the recurrence and metastasis of tumors and achieve better chemotherapy effects. Animal studies have shown that propofol inhibits tumor growth and improves survival in mice.

Our study found for the first time that anesthetic sedative drug propofol can inhibit self-renewal of BCSCs. To further investigate the mechanisms that inhibit self-renewal of BCSCs, we examined some of the conserved signaling pathways commonly found in cancer stem research. Compared with the control group, the Hedgehog pathway protein activation was lowest in the T24 and 5637 tumor cell lines after propofol incubation, and the downstream transcription factors Gli1 and Gli2 were abnormally reduced. It is suggested that propofol may inhibit the self-renewal of BCSCs by inhibiting the Hedgehog pathway, which plays an important role in stem cells from a variety of tumors[33–35]. Further study will be conducted in our laboratory to explore how propofol regulates Hedgehog pathway in BCSCs.

A number of studies have shown that other anesthetics have a certain effect on tumor cells. The effects of opioids on tumor growth and metastasis are controversial and may be related to factors such as drug concentration, time of action, and type of tumor[36–38]. Sevoflurane is thought to inhibit cell-mediated immunity, induce apoptosis of T lymphocytes, and promote proliferation, invasion, and migration of tumor cell[39,40]. By understanding the effects of various anesthetics on the biological behavior of tumors and their effects on CSCs, this will help us optimize the selection and management of anesthetic drugs and further find effective tumor-targeted therapeutic drugs.

Ethical Approval

This study was approved by Peking University Third Hospital Medical Science Research Ethics Committee, Beijing, China.

Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with the Institutional Animal Care Guidelines of Peking University Third Hospital, Beijing, China.

Statement of Informed Consent

Informed consent was obtained from all patients in this study.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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