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Anti-proliferation and anti-metastatic effects of sevoflurane on human osteosarcoma U2OS and Saos-2 cells

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ABSTRACT

Sevoflurane is a commonly used anesthetic in cancer surgery. However, little information is available about the effects of sevoflurane on the osteosarcoma cells. The present study explored the effects of sevoflurane on the proliferation, migration and invasion of osteosarcoma cells, as well as the underlying mechanism. Human osteosarcoma cell lines U2OS and Saos-2 were pretreated with different concentrations (2.5%, 5%, or 10%) or different times (0, 2, 4 or 6 h) of sevoflurane. Cell viability, proliferation, migration, invasion and apoptosis were analyzed by MTT assay, Ki67 staining, colony formation assay, wound healing scratch assay, transwell assays and Annexin V-FITC/PI staining. Expression of key factors related to proliferation, migration, apoptosis, epithelial-mesenchymal transition (EMT) process and PI3K/AKT pathway were evaluated by western blotting. We found that sevoflurane significantly inhibited U2OS and Saos-2 cell viability, proliferation, migration and invasion, but induced cell apoptosis. Moreover, sevoflurane obviously inactivated PI3K/AKT pathway in U2OS and Saos-2 cells. Activator of PI3K/AKT pathway, insulin-like growth factor-1 (IGF-1) notably weakened the effects of sevoflurane on U2OS and Saos-2 cell proliferation, migration and apoptosis. Besides, sevoflurane suppressed EMT process in U2OS and Saos-2 cells. To sum up, our results evidenced that sevoflurane exerted anti-proliferative and anti-metastatic effects on osteosarcoma cells by inactivating PI3K/AKT pathway.

1. Introduction

Osteosarcoma is a primary bone tumor with high rate of malignity, high degree of incidence and high frequency of recurrence and metastasis, especially among children and adolescents (Chen et al., 2013; Tang et al., 2014). Surgery remains the first-line treatment method for patients with solid tumors, including osteosarcoma (Gerrand and Rankin, 2014). Surgical resection combined with adjuvant therapy is effective in treatment of osteosarcoma (Carrle and Bielack, 2006). With the advancement of treatment (including chemotherapy and surgery), the survival rate for osteosarcoma has increased to 65–75% (Xu et al., 2013a; Zhao et al., 2012). But the 5-year survival rate of patients is still very low due to the occurrence of tumor recurrence and metastasis (Weidle et al., 2016). It is essential to better understand the pathogenesis of osteosarcoma and identify new therapeutic strategies and medicines for osteosarcoma.

The influence of anesthesia on the tumor growth and recurrence after surgery has been paid more and more attention in recent years (Kumar, 2016; Yeager and Rosenkranz, 2010). Anesthesia can affect the outcomes of cancer patients by regulating neuroendocrine stress response and immune system (Lee et al., 2004). Recent study has suggested that the volatile anesthetics, such as sevoflurane and desflurane, have impacts on invasion and migration of some tumor cells (Preckel and Bolten, 2005).

Sevoflurane has been reported to inhibit proliferation and migration of various cancer cells (Ecimovic et al., 2013). In the study by Kvolik et al., the anti-proliferative effect of sevoflurane on colon cancer SW620 and Caco-2 cells has been demonstrated (Kvolik et al., 2005). Other study showed that sevoflurane inhibit the infiltration of lung cancer cells (Liang et al., 2012a) and prevent the migration of glioma cells (Yi et al., 2016). Moreover, researchers also proved that sevoflurane and desflurane impaired neutrophil matrix metalloproteinase (MMP)-9 release and reduced subsequent migration of colon cancer cells (Mülleredenborn et al., 2012). In addition, the role of anesthetics in the regulation of biological processes in tumor cells has also been studied (Mao et al., 2013). However, the role of sevoflurane in osteosarcoma

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has not been studied. Therefore, in the present study, we determined the effects of sevoflurane on the viability, proliferation, migration, invasion and EMT process of osteosarcoma U2OS and Saos-2 cells. The possible internal signaling pathways were also explored.

2. Materials and methods

2.1. Cell lines

Human osteosarcoma cell lines, U2OS and Saos-2, were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). U2OS and Saos-2 cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Gibco, Life Technologies, CA, USA) containing with 10% (*v*/v) heat-inactivated fetal bovine serum (FBS, Hyclone, UT, USA) and 100 U/ml benzyl penicillin-100 μg/ml streptomycin solution (Gibco, Life Technologies, CA, USA) at 37 °C in a humidified incubator (Thermo Fisher Scientific, Waltham, MA, USA) with 5% CO_2.

Insulin-like growth factor-1 (IGF-1) was obtained from Novoprotein Scientific Inc. (Shanghai, China). For activation of PI3K/AKT pathway, 25 ng/ml IGF-1 was added into culture medium simultaneously with sevoflurane treatment.

2.2. Pretreatment with sevoflurane

U2OS and Saos-2 cells were seeded into 24 or 96-well plate with 3×10^4 or 1×10^4 cells each well overnight. Following with the experimental protocol described previously (Hurmath et al., 2016), cell plates containing with U2OS or Saos-2 cells were placed into a sealed modular incubator chamber (Thermo Fischer Scientific, Waltham, MA, USA) with inlet and outlet connectors. The inlet connector was lined to the anesthesia machine and sevoflurane (Sigma-Aldrich, St Louis, MO, USA) was added into the chamber by a sevoflurane vaporizer (Drager Vapor 2000, Lubeck, Germany). In our experiments, cells were exposed to 2.5%, 5% or 10% concentrations of sevoflurane for 0, 2, 4 or 6 h in air oxygen mixture with fraction of inspiration O_2 (FiO_2) of 0.45–0.55. Sevoflurane concentration in sealed modular incubator chamber was monitored by an anesthetic gas monitor (RIKEN, Kobe, Japan). Cells in the control group were only exposed to air oxygen mixture with FiO_2 of 0.45–0.55.

2.3. MTT assay

Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay. Briefly, U2OS and Saos-2 cells were seeded into 96-well plate and exposure to 0% or 2.5% sevoflurane for 0, 2, 4 or 6 h. Then 20 μl MTT solution (2.5 mg/ml in PBS) was added into each well of plate followed by incubated for other 4 h at 37 °C with 5% CO_2. After that, the cell culture medium was removed and 150 μl dimethyl sulfoxide (DMSO) was added into each well. The absorbance at 570 nm of each well was recorded using Bio-Tek multi-well plate reader (Bio-Tek, Winooski, VT, USA).

3. Ki67 staining assay

Cell proliferation was detected using Ki67 staining assay. Briefly, U2OS and Saos-2 cells were seeded into 24-well plate and treated by 0%, 2.5%, 5% or 10% sevoflurane for 6 h. Then, cells in each group were collected and washed with phosphate buffered saline (PBS) for three times. After that, each sample (200 μl) was stained with 2 μl Ki67 staining solution at 37 °C in the dark for 25 min. The Ki67-positive (+) cells in each group was quantified using FlowJo software (Tree Star Inc., Ashland, OR, USA). Data were analyzed using FCS Express software (De Novo Software, Los Angeles, CA, USA).

3.1. Colony formation assays

Colony formation assay was performed to analyze the long-term proliferative abilities of U2OS and Saos-2 cells. After different concentrations of sevoflurane treatment for 6 h, approximately 500 cells of each group were added into a 6-well plate. The medium was changed every other day until the visible colonies formed. After that, cells were washed with PBS for three times, fixed using 4% paraformaldehyde solution for 20 min at 4 °C and stained using 10% Giemsa for 25 min. Colonies containing ≥50 cells were counted under a microscope. Colony formation (%) was quantified using mean colonies number in treatment group/mean colonies number in control group × 100%.

3.2. Wound healing migration assay

The changes of migration of U2OS and Saos-2 after sevoflurane treatment were investigated using wound healing migration assay as previously described with little modification (Hurmath et al., 2016). U2OS and Saos-2 cells in each group after different concentrations of sevoflurane treatment for 6 h were wounded using a 200 μl sterile pipette tip. Then, cells in each group were washed with PBS for three times to remove the suspended cells. After that, cells were cultured into serum-free medium for 2 days. Cell migration (%) was calculated by average migrated cells in treatment group/average migrated cells in control group × 100%.

3.3. Cell invasion assay

Cell invasion was tested using a modified two-chamber transwell invasion assay with 8 mm PET membranes (Millipore, Bedford, MA, USA) in line with the manufacturer's instruction. Briefly, after 0%, 2.5%, 5% or 10% sevoflurane treatment for 6 h, 1×10^4 U2OS or Saos-2 cells were re-suspended in 200 μl serum-free medium and seeded into the upper chamber. In the lower chamber, 600 μl medium containing with 10% FBS was added and served as a chemo-attractant. After incubation at 37 °C for 48 h, non-invaded cells were removed carefully using a cotton swab, while invaded cells adhering to the lower membrane surface were fixed with methanol, stained using crystal violet and counted under a microscope (Nikon, Japan). Cell invasion was quantified using average number of invading cells in each group from five randomly chosen fields.

3.4. Apoptosis assay

Apoptosis analysis was performed using Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China) according to the manufacturer's protocol. Briefly, U2OS or Saos-2 cells were seeded into 24-well plate with 3×10^4 each well and treated by 0%, 2.5%, 5% or 10% sevoflurane for 6 h. Then, cells in each group were harvested and washed with PBS for three times. After that, 100 μl cells were mixed with 100 μl Annexin V-FITC/PI solution at 37 °C for 20 min in the dark. Flow cytometer (Beckman Coulter, USA) was used to differentiate apoptotic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive). Data were analyzed using FCS Express software (De Novo Software, Los Angeles, CA, USA).

3.5. Western blotting assay

Total proteins in U2OS or Saos-2 cells after relevant treatment were extracted using RIPA lysis and extraction buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with protease inhibitors (Roche, Basel, Switzerland) in line with the manufacturer's instruction. The protein concentrations were quantified using BCA™ Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Western blotting system was established using Bio-Rad Bis-Tris Gel system (Bio-Rad, Shanghai, China) according to the manufacturer's instructions. Protein

samples were electrophoresed and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA), which were incubated with primary antibodies. Primary antibodies were prepared in 1% bovine serum albumin (BSA, Sigma-Aldrich, St Louis, MO, USA) solution at a dilution of 1:1000. Anti-p21 antibody (ab109520), Anti-proliferating cell nuclear antigen (PCNA) antibody (ab18197), Anti-CyclinD1 antibody (ab134175), Anti-Bcl-2 antibody (ab32124), Anti-cleaved caspase 8 antibody (ab32397), Anti-cleaved caspase 3 antibody (ab13847), Anti-phospho-AKT antibody (ab38449), Anti-AKT antibody (ab8805), Anti-phospho-PI3K antibody (ab182651), Anti-PI3K antibody (ab86714), Anti-N-cadherin antibody (ab18203), Anti-Fibronectin antibody (ab32419), Anti-β-catenin antibody (ab32572), Anti-E-cadherin antibody (ab76055) and Anti-GAPDH antibody (ab8245) were all obtained from Abcam Biotechnology (Cambridge, MA, USA). Primary antibody was incubated with the PVDF membrane at 4 °C overnight, followed by wash and incubation with Goat Anti-Rabbit (or Anti-mouse) IgG H&L (HRP) secondary antibodies (ab205718, ab205719, Abcam Biotechnology, Cambridge, MA, USA) for 1 h at room temperature in the dark. After rinsing, the PVDF membrane-carried blots and antibodies were transferred into Bio-Rad ChemiDoc™ XRS system, and then 200 μl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. Protein signals and the intensities of the bands were captured using Image Lab™ Software (Bio-Rad, Shanghai, China).

3.6. Statistical analysis

All experiments were performed three times in triplicate. Results of multiple experiments were presented as mean ± standard deviation (SD). Statistical analyses were performed using SPSS 19.0 statistical software. *P*-values between different groups were calculated using Student's *t*-test. A value of $P < 0.05$ was considered to indicate a statistically difference result.

4. Results

4.1. Sevoflurane inhibits the viability and proliferation of osteosarcoma cells

Viabilities of U2OS and Saos-2 cells after sevoflurane treatment for 2, 4 or 6 h were measured by MTT assay. As presented in Fig. 1A, in the presence of sevoflurane (2.5%), viabilities of U2OS and Saos-2 cells were remarkably decreased in a time-dependent manner ($P < 0.05$ or $P < 0.01$). For U2OS cells, the viabilities were reduced to 8.52 ± 1.89%, 22.84 ± 2.69% and 30.26 ± 3.52% for 2, 4 and 6 h, respectively. For Saos-2 cells, the viabilities were reduced to 10.80 ± 1.20%, 26.14 ± 2.28% and 39.09 ± 3.75% for 2, 4 and 6 h, respectively. The incubation time of 6 h was chosen for subsequent experiments. The effects of sevoflurane (at 2.5%, 5%, and 10% concentration) treatment for 6 h on cell proliferation were detected by Ki67 staining and colony formation. As shown in Fig. 1B, there were dose-dependent decreases in the percentages of Ki67-positive cells in both U2OS and Saos-2 cells after sevoflurane treatment. In U2OS cells, the rates Ki67-posotive cells were decreased to 27.33 ± 4.26%, 23.67 ± 4.66% and 16.56 ± 3.75% with an increasing dose of sevoflurane at 2.5% ($P < 0.05$), 5% ($P < 0.05$), and 10% ($P < 0.01$), when compared to control group cells (41.85 ± 5.56%). In Saos-2 cells, there was no significant decrease of Ki67-positive cells in sevoflurane 2.5% group (26.36 ± 3.65%) when compared to control group (35.23 ± 5.12%). But, an obvious decrease of Ki67-positive cells was seen at 5% (17.67 ± 2.85%, $P < 0.05$) and 10% (10.33 ± 2.25%, $P < 0.01$) concentrations. Colony formation assay displayed that sevoflurane treatment significantly reduced the colony numbers of U2OS and Saos-2 in dose-dependent manners ($P < 0.01$, Fig. 1C). For U2OS cells, the rates of colony numbers were reduced to 82 ± 4.15% after 2.5% sevoflurane treatment (no significant), 62.33 ± 3.26% after 5% sevoflurane treatment ($P < 0.01$) and 55 ± 4.6% after 10% sevoflurane treatment ($P < 0.01$). For Saos-2 cells, the rates of colon numbers were reduced to 77.67 ± 2.66 after 2.5% sevoflurane treatment ($P < 0.01$), 60.73 ± 4.56% after 5% sevoflurane treatment ($P < 0.01$) and 50 ± 4.26% after 10% sevoflurane treatment ($P < 0.01$). In addition, western blotting analysis was performed to detect the effects of 2.5%, 5% or 10% sevoflurane treatments on the expressions of p-21, PCNA, and CyclinD1 in U2OS and Saos-2 cells (Fig. 1D). In both the cell lines, there was a dose-dependent increase of p-21 expression (Fig. 1E and F, $P < 0.05$ or $P < 0.01$), and dose-dependent decreases of the expressions of PCNA and CyclinD1 ($P < 0.05$ or $P < 0.01$) after 2.5%, 5% or 10% sevoflurane treatments for 6 h. These results show that sevoflurane inhibits the viability and proliferation of osteosarcoma cells.

4.2. Sevoflurane inhibits the migration and invasion of osteosarcoma cells

Cell migration and invasion have been proved to play important roles in tumor metastasis (Xu et al., 2013b). Next, we investigated the effects of sevoflurane on the migration and invasion of osteosarcoma U2OS and Saos-2 cells using wound healing assay and transwell invasion assay, respectively. As denoted in Fig. 2A, sevoflurane at 2.5% concentration had no obvious effects on the migration of U2OS and Saos-2 cells, while sevoflurane at 5% and 10% concentrations significantly inhibited the migration of U2OS and Saos-2 cells in dose-dependent manners ($P < 0.01$). Compared to the cells cultured in normal condition, the migration of U2OS cells were decreased to 62.67 ± 3.57% and 53.26 ± 3.85%, respectively, after 5% and 10% sevoflurane treatment; the migration of Saos-2 cells were decreased to 62.35 ± 3.35% and 51 ± 4.06%, respectively, after 5% and 10% sevoflurane treatment. In addition, sevoflurane also inhibited the invasion of U2OS and Saos2 cells compared to the control cells (Fig. 2B). For U2OS cells, the invasive cell numbers were reduced to 59 ± 2.53% ($P < 0.05$) and 45.26 ± 3.12% ($P < 0.01$) after 5% and 10% sevoflurane treatment, relative to control group (80.33 ± 3.83%). For Saos-2 cells, the invasive cell numbers were reduced to 45.46 ± 2.37% ($P < 0.01$) after 10% sevoflurane treatment, relative to control group (87.67 ± 4.66%). These data indicates that sevoflurane reduces osteosarcoma cell migration and invasion. We also determined the effects of sevoflurane at 2.5%, 5%, and 10% concentrations on the expression of metastasis-related protein MMP-9. As shown in Fig. 2C, 5% sevoflurane treatment inhibited the expressions of MMP-9 by 0.52 ± 0.06 and 0.55 ± 0.05 folds in U2OS and Saos-2 cells, respectively ($P < 0.05$). 10% sevoflurane treatment reduced the expression of MMP-9 by 0.33 ± 0.06 and 0.33 ± 0.05 folds in U2OS and Saos-2 cells, respectively ($P < 0.01$). These findings suggest that sevoflurane inhibits the migration and invasion of osteosarcoma cells.

4.3. Sevoflurane promotes apoptosis of osteosarcoma cells

The apoptosis of U2OS and Saos-2 cells were measured using Annexin V-FITC/PI staining and flow cytometry analysis. As presented in Fig. 3A, the rate of apoptotic U2OS cells was significantly increased to 23.36 ± 2.15% after 5% sevoflurane stimulation ($P < 0.01$) and increased to 35.78 ± 4.42% after 10% sevoflurane stimulation ($P < 0.01$), compared to control group (9.05 ± 2.89%). Similarly, the rate of apoptotic Saos-2 cells was dramatically increased to 31.58 ± 7.63% after 10% sevoflurane stimulation ($P < 0.05$), compared to control group (7.33 ± 3.26%). To confirm this, we also measured the expression of apoptotic-related proteins (Bcl2, cleaved-caspase 3 and cleaved-caspase 8) using western blotting. As displayed in Fig. 3B, 5% and 10% sevoflurane treatment remarkably decreased the expression of Bcl2 ($P < 0.05$ for 5% and $P < 0.01$ for 10% in both U2OS and Saos-2 cells), and increased the expressions of cleaved-caspase 3 and cleaved-caspase 8 ($P < 0.05$ for 5% and $P < 0.01$ for 10% in U2OS cells; $P < 0.01$ for 10% in Saos-2 cells). These results suggest that sevoflurane promotes the apoptosis of osteosarcoma cells.

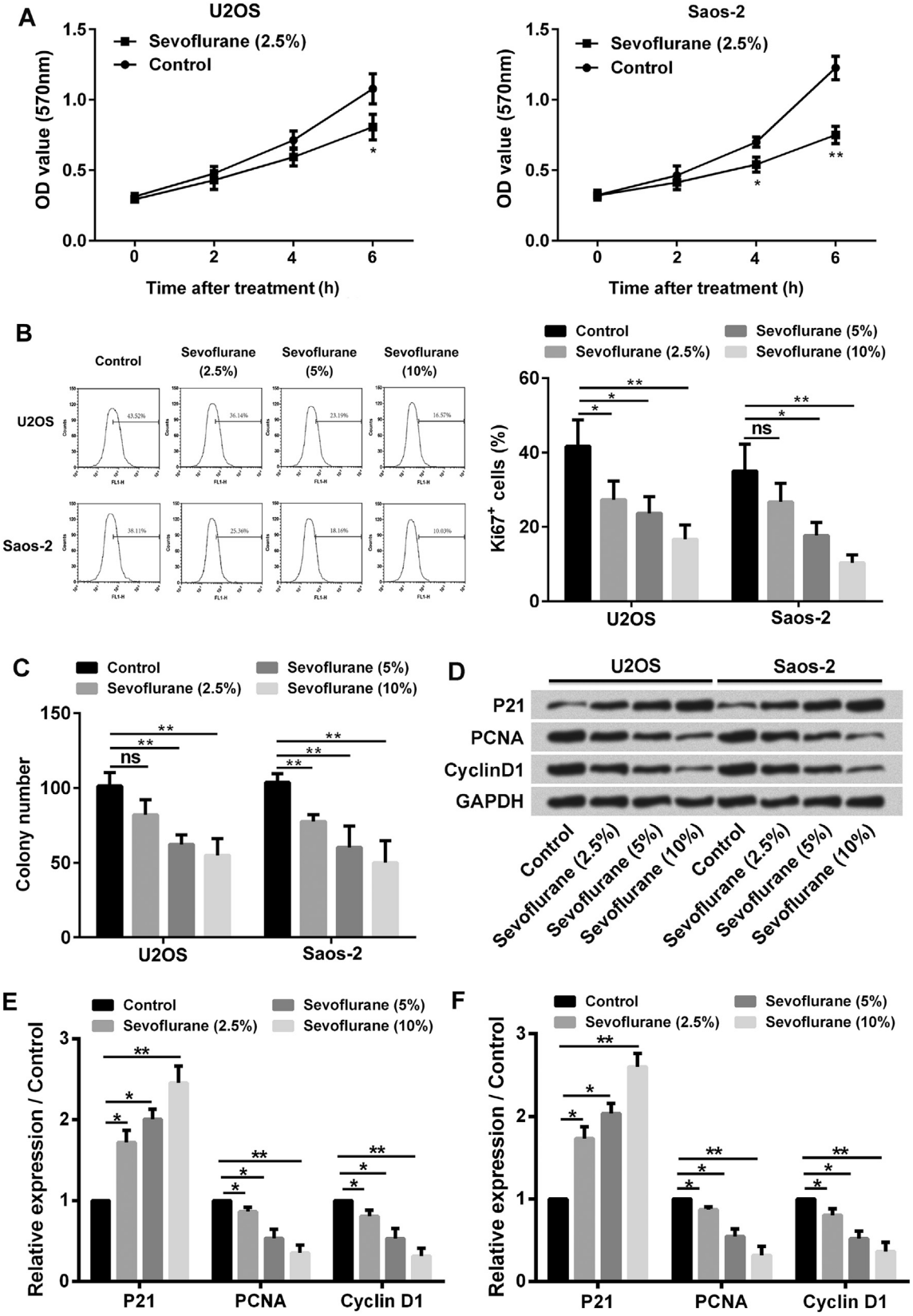

Fig. 1. Sevoflurane inhibits the viability and proliferation of osteosarcoma cells.
(A) Viabilities of U2OS and Saos-2 cells after sevoflurane (2.5%) treatment for 2, 4 and 6 h were measured by MTT assay. (B) Ki67 staining was used to detect the proliferation of U2OS and Saos-2 cells after sevoflurane (2.5%, 5% and 10%) treatment for 6 h. (C) Colony formation assay was performed to detect the effects of sevoflurane (2.5%, 5% and 10%) treatment for 6 h on long-term proliferative potential of U2OS and Saos-2 cells. (D-F) Western blotting was used to analyze the protein expressions of p-21, PCNA, and CyclinD1 in U2OS and Saos-2 cells after sevoflurane (2.5%, 5% and 10%) treatment for 6 h. NS: Not significant; PCNA: Proliferating cell nuclear antigen. NS: No significant. $^{*}P < 0.05$, $^{**}P < 0.01$.

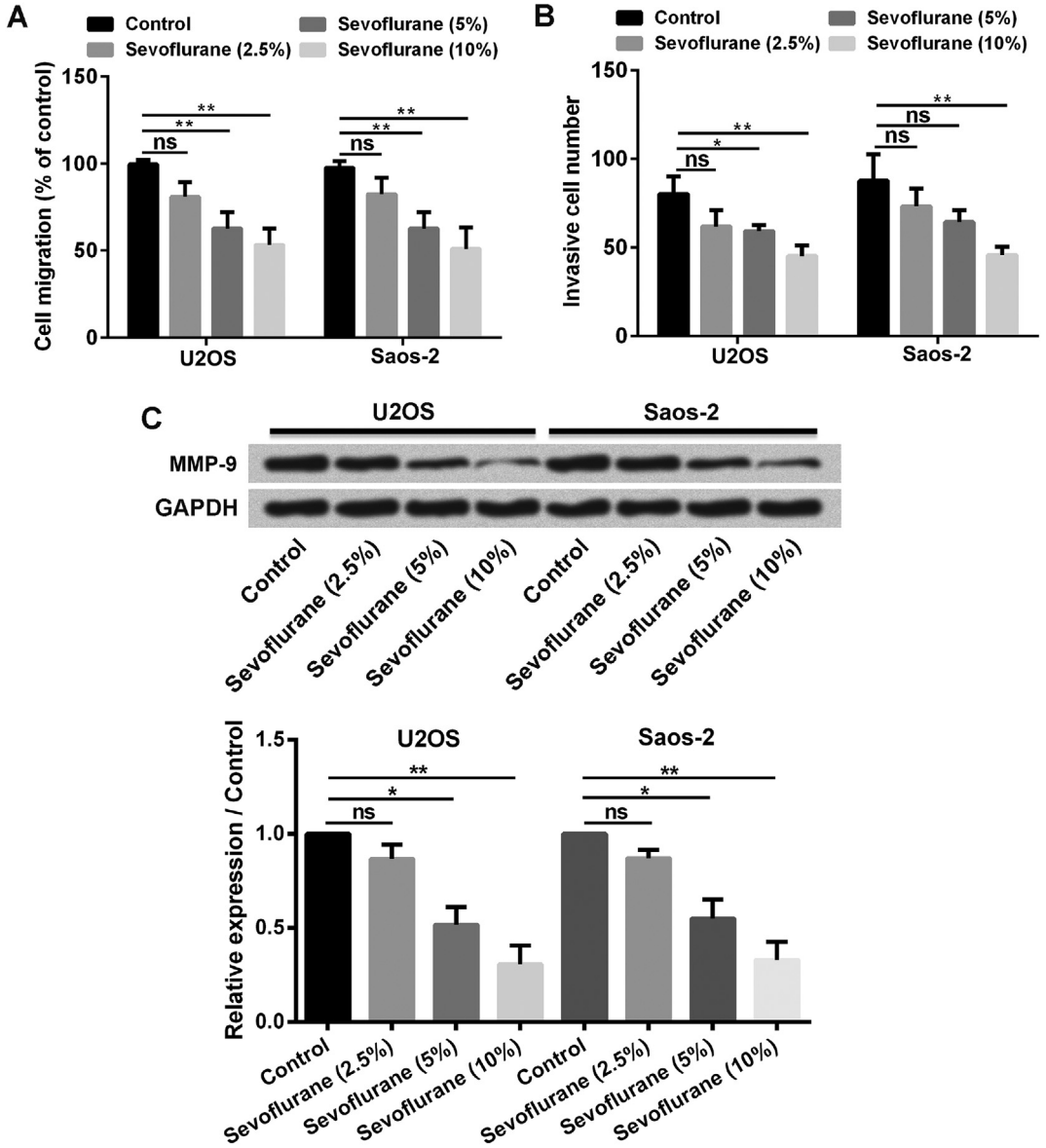

Fig. 2. Sevoflurane inhibits the migration and invasion of osteosarcoma cells.
(A) Effects of sevoflurane at 2.5%, 5% and 10% treatment for 6 h on the migration of U2OS and Saos-2 cells were measured by wound healing assay. (B) Effects of sevoflurane at 2.5%, 5% and 10% treatment for 6 h on the invasion of U2OS and Saos-2 cells were measured by transwell invasion assay. (C) Western blotting was used to measure the protein expressions of MMP-9 in U2OS and Saos-2 cells after sevoflurane (2.5%, 5% and 10%) treatment for 6 h. NS: Not significant; MMP-9: Matrix metallopeptidase 9. NS: No significant. *$P < 0.05$, **$P < 0.01$.

4.4. Sevoflurane inactivates PI3K/AKT pathway in U2OS and Saos-2 cells

The PI3K/AKT pathway is one of the most major signaling pathways in cells associated with cancer progression and invasion (Liang et al., 2012a; Mao et al., 2013). Therefore, we investigated the effects of sevoflurane at 2.5%, 5%, and 10% concentrations on the expressions of p-PI3K, PI3K, p-AKT and AKT in U2OS and Saos-2 cells. Results showed that the expression rates of p/t-PI3K and p/t-AKT were both decreased in U2OS and Saos-2 cells after 5% or 10% sevoflurane treatment compared to the control group (Fig. 4A, $P < 0.05$ for 5%, $P < 0.01$ for 10%). For example, 5% and 10% sevoflurane treatment reduced the expression rates of p/t-PI3K by 0.51 ± 0.05 and 0.34 ± 0.05 folds, respectively, and reduced the expression rates of p/t-AKT by 0.53 ± 0.05 and 0.36 ± 0.05 folds in U2OS cells. Next, we determined the effects of 5% sevoflurane treatment for 2, 4 or 6 h on the expressions of p-PI3K, PI3K, p-AKT and AKT in U2OS and Saos-2 cells. As shown in Fig. 4B, the expression rates of p/t-PI3K and p/t-AKT were both decreased in U2OS and Saos-2 cells after 5% sevoflurane treatment for 4 or 6 h, compared to the control group ($P < 0.05$ for 4 h, for $P < 0.01$ 6 h). Moreover, activator of PI3K/AKT pathway, IGF-1, was added into our experiments, the results of Fig. 4C displayed that 10% sevoflurane treatment-induced decreases of p/t-PI3K and p/t-AKT expression rates in both U2OS and Saos-2 cells were significantly reversed by 25 ng/ml IGF-1 incubation ($P < 0.01$). These results imply that sevoflurane exert anti-cancer effects on osteosarcoma cells might be via suppressing the PI3K/AKT pathway.

4.5. Inactivation of PI3K/AKT pathway plays critical roles in the anti-cancer effects of sevoflurane on osteosarcoma cells

Subsequently, we further explored the effects of IGF-1 incubation on the 10% sevoflurane treatment-induced osteosarcoma U2OS and Saos-2 cell proliferation and migration inhibition, as well as cell apoptosis. Fig. 5A showed that 25 ng/ml IGF-1 incubation notably increased the

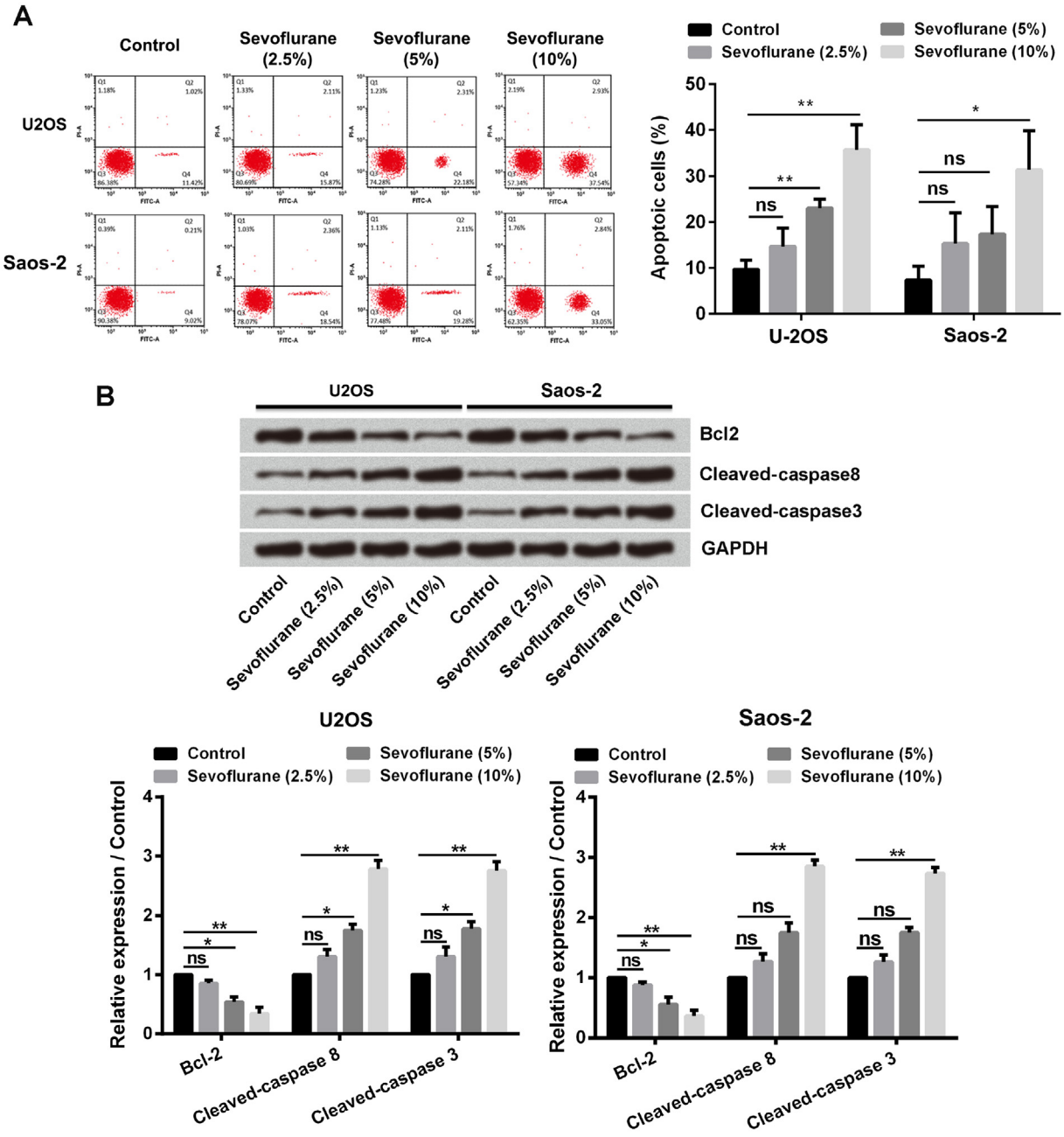

Fig. 3. Sevoflurane promotes apoptosis of osteosarcoma cells.
(A) Annexin V-FITC/PI staining and flow cytometry was performed to measure apoptosis of U2OS and Saos-2 cells after sevoflurane (2.5%, 5% and 10%) treatment for 6 h. (B) Western blotting was used to measure the expressions of apoptosis-related proteins (Bcl2, cleaved caspase 3 and cleaved caspase 8) in U2OS and Saos-2 cells after sevoflurane (2.5%, 5% and 10%) treatment for 6 h. NS: No significant. *$P < 0.05$, **$P < 0.01$.

colony number of U2OS cells to 78.52 ± 2.26%, relative to 51.33 ± 3.13% in single 10% sevoflurane treatment ($P < 0.05$) and enhanced the colony number of Saos-2 cells to 69.65 ± 0.73%, relative to 46.67 ± 5.02% in single 10% sevoflurane treatment ($P < 0.05$). Moreover, similar results were found in Fig. 5B, which presented that 25 ng/ml IGF-1 incubation significantly mitigated the 10% sevoflurane treatment-induced U2OS and Saos-2 cell migration reduction. For U2OS cells, the migration was enhanced to 76 ± 3.18%, relative to 52.72 ± 2.15% in single 10% sevoflurane treatment ($P < 0.05$). For Saos-2 cells, the migration was enhanced to 80.62 ± 2.53%, relative to 49.32 ± 4.26% in single 10% sevoflurane treatment ($P < 0.05$). Besides, the 10% sevoflurane treatment-induced U2OS and Saos-2 cell apoptosis were also alleviated by 25 ng/ml IGF-1 incubation (Fig. 5C), which displayed that the rate of apoptotic U2OS cells was reduced to 16.28 ± 10.58%, compared to 35.62 ± 7.24% in single 10% sevoflurane treatment ($P < 0.05$) and the rate of apoptotic Saos-2 cells was decreased to 14.57 ± 9.78%, compared to 32.67 ± 11.69% in single 10% sevoflurane treatment ($P < 0.05$). These above findings imply that inactivation of PI3K/AKT pathway plays critical roles in the anti-cancer effects of sevoflurane on osteosarcoma cells.

4.6. Sevoflurane inhibits epithelial-mesenchymal transition (EMT) process in osteosarcoma U2OS and Saos-2 cells

Lastly, we explored the effects of sevoflurane at 2.5%, 5%, and 10% concentration on EMT process in osteosarcoma U2OS and Saos-2 cells by measuring the expression levels of epithelial markers (E-cadherin and β-catenin) and mesenchymal markers (Fibronectin and N-cadherin)

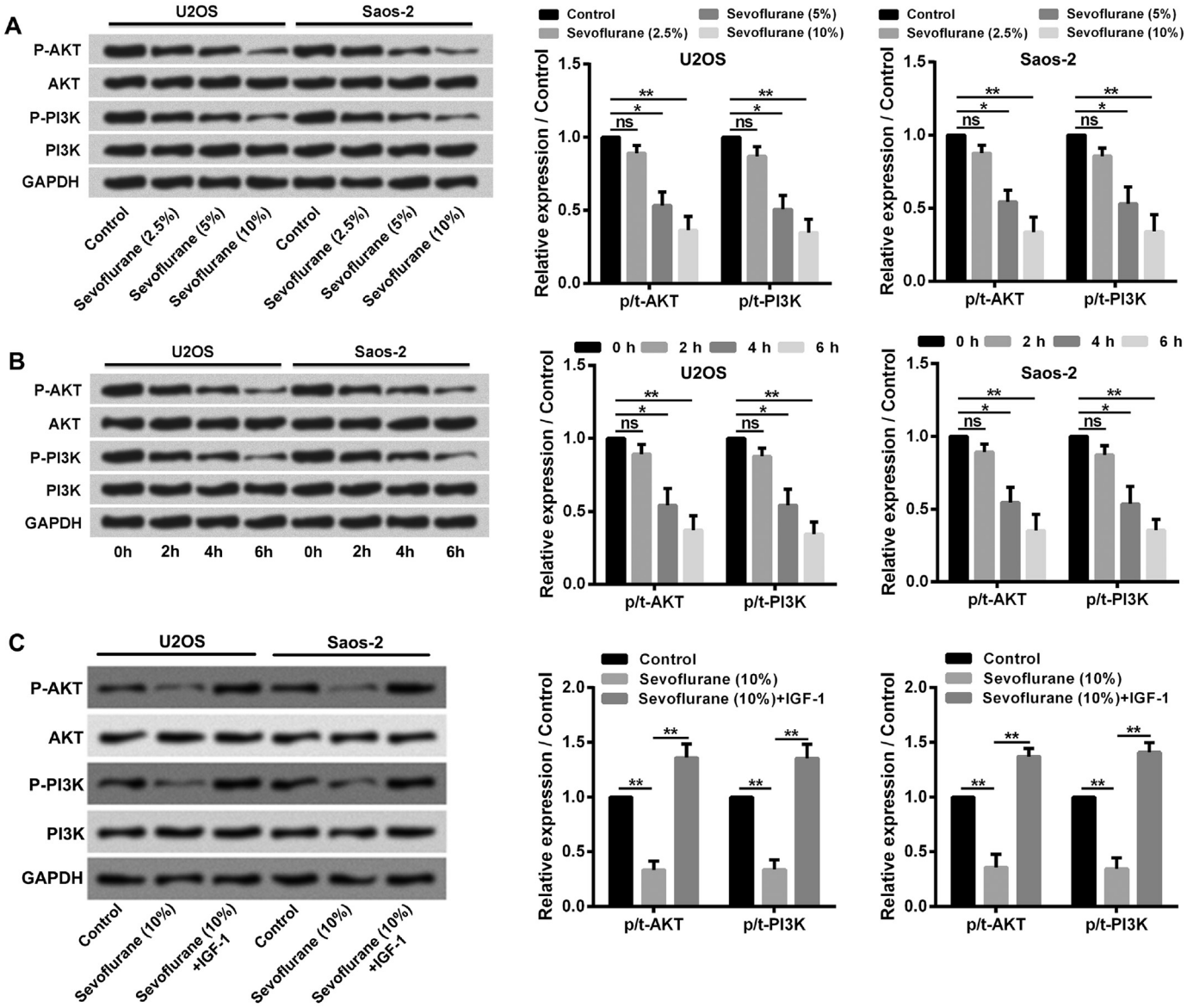

Fig. 4. Sevoflurane inactivated PI3K/AKT pathway in osteosarcoma cells.
(A) The expressions of p-PI3K, p-AKT, PI3K, and AKT in U2OS and Saos-2 cells were detected by western blotting analysis following exposure to 2.5%, 5% or 10% sevoflurane treatment for 6 h. (B) Effects of 5% sevoflurane treatment for 2, 4 and 6 h on the levels of p-PI3K, p-AKT, PI3K, and AKT in U2OS and Saos-2 cells were detected by western blotting analysis. (C) The expressions of p-PI3K, p-AKT, PI3K, and AKT in U2OS and Saos-2 cells after 10% sevoflurane and/or 25 ng/ml IGF-1 treatment were detected by western blotting. PI3K: Phosphatidylinositol 3-kinase; AKT: Protein kinase 3, IGF-1: Insulin-like growth factor-1. NS: No significant. $^{*}P < 0.05$, $^{**}P < 0.01$.

using western blotting. For U2OS cells, 5% sevoflurane treatment down-regulated the expression levels of N-cadherin and Fibronectin by 0.56 ± 0.03 and 0.54 ± 0.03 folds, respectively, but up-regulated the expression levels of E-cadherin and β-catenin by 1.75 ± 0.02 and 1.74 ± 0.03 fold, respectively, compared to control group (Fig. 6A, $P < 0.05$). 10% sevoflurane treatment down-regulated the expression levels of N-cadherin and Fibronectin by 0.35 ± 0.03 and 0.33 ± 0.02 folds, respectively, but up-regulated the expression levels of E-cadherin and β-catenin by 2.87 ± 0.02 and 2.85 ± 0.03 folds, respectively, compared to control group (Fig. 6B; $P < 0.01$). Similar results were found in Saos-2 cells ($P < 0.05$ for 5% concentration and $P < 0.01$ for 10% concentration). Moreover, we also measured the expression levels of these proteins following the exposure to 5% sevoflurane treatment for 2, 4 or 6 h. For U2OS cells, results presented that 5% sevoflurane treatment for 4 h down-regulated the expression levels of N-cadherin and Fibronectin by 0.52 ± 0.03 and 0.54 ± 0.03 folds, respectively, but up-regulated the expression levels of E-cadherin and β-catenin by 1.72 ± 0.04 and 1.74 ± 0.03 folds, respectively, compared to control group (Fig. 6B, $P < 0.05$). 5% sevoflurane treatment for 6 h down-regulated the expression levels of N-cadherin and Fibronectin by 0.32 ± 0.03% and 0.33 ± 0.03%, respectively, but up-regulated the expression levels of E-cadherin and β-catenin by 2.81 ± 0.03% and 2.84 ± 0.03%, respectively, compared to control group ($P < 0.01$). Similar results were also presented in Saos-2 cells ($P < 0.05$ for 4 h incubation and $P < 0.01$ for 6 h incubation). These findings indicate that sevoflurane inhibits the EMT process in osteosarcoma cells.

5. Discussion

Recent studies suggest that anesthetics potentially affect cancer progression after surgery (Meng et al., 2006; Shih et al., 2012). But, little information is available about the direct effects of specific

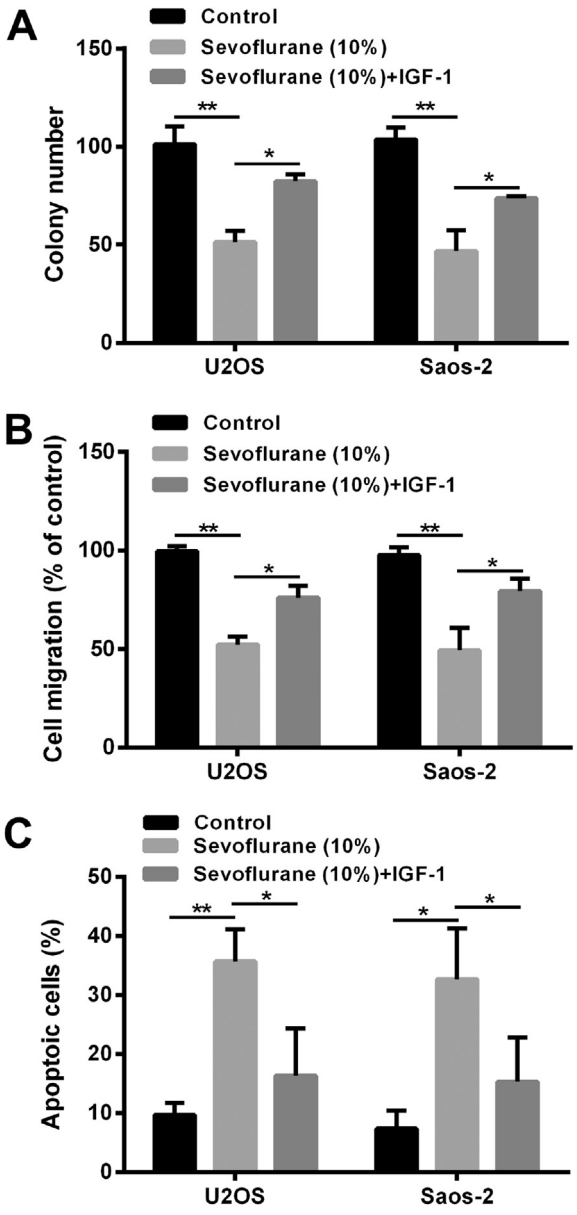

Fig. 5. Inactivation of PI3K/AKT pathway plays critical roles in the anti-cancer effects of sevoflurane on osteosarcoma cells.
(A) Colony formation assay was performed to detect the effects of sevoflurane (10%) and/or IGF-1 (25 ng/ml) treatment for 6 h on long-term proliferative potential of U2OS and Saos-2 cells. (B) Migration of U2OS and Saos-2 cells were tested using wound healing assay after sevoflurane (10%) and/or IGF-1 (25 ng/ml) treatment for 6 h. (C) Apoptosis of U2OS and Saos-2 cells were tested using Annexin V-FITC/PI staining and flow cytometry after sevoflurane (10%) and/or IGF-1 (25 ng/ml) treatment for 6 h. IGF-1: Insulin-like growth factor-1. $^{*}P < 0.05$, $^{**}P < 0.01$.

anesthetic agents on osteosarcoma cell proliferation and invasion. The present study aimed to evaluate the effects of sevoflurane on the proliferation, migration, invasion, apoptosis and EMT process of osteosarcoma cells. The clinical use of sevoflurane was: induction of 8%, oxygen flow rate of 6 L/min; maintenance of 2.5%–3%, oxygen flow rate of 2 L/min. In the experiment of Liang et al., they had chosen 1.7%, 3.4% and 5.1% of sevoflurane to treat lung carcinoma A549 cells (Liang et al., 2011). In the experiment of Hurmath et al., they had chosen 2.5% of sevoflurane to treat glioma U87MG cells (Hurmath et al., 2016). In our experiments, to simulate the clinical anesthesia setting, osteosarcoma U2OS and Saos-2 cells were treated with three different concentrations (2.5%, 5% and 10%) of sevoflurane. We found that sevoflurane inhibits the viabilities and proliferation of U2OS and Saos-2 cells in dose- and time-dependent manners. These findings were quite consistent with the previous study, which indicated that sevoflurane inhibits the migration and invasion of glioma and lung adenocarcinoma cells (Liang et al., 2012b; Yi et al., 2016).

Osteosarcoma cells are derived from malignant bone tumors. The degradation of the extracellular matrix (ECM) contributes to the migration and invasion of tumor cells, including osteosarcoma cells (Snyder and Greenberg, 2010). MMPs could regulate the level of ECM (Guha et al., 2009). In osteosarcoma cells, the most prominent alteration is the positive labeling results for MMP-9 and collagen-X, which can be detected in all osteosarcoma cell lines but not in normal osteoblasts (Tavare et al., 2012). Several studies suggest that MMPs, especially MMP-9, play vital roles in the cell growth and tumor metastasis (Hegedüs et al., 2006; Pautke et al., 2004). Previous study had demonstrated that MMP-9 was a potential biomarker for osteosarcoma, the expression of MMP-9 in osteosarcoma tissues was higher than in other normal tissues (Patterson et al., 2001). Therefore, we evaluated the effect of sevoflurane on the migration and invasion of U2OS and Saos-2 cells, as well as the expression of MMP-9 in U2OS and Saos-2 cells. We found that sevoflurane suppressed the migration and invasion of U2OS and Saos-2 cells in concentration-dependent manners and down-regulated the expression of MMP-9 in both U2OS and Saos-2 cells in a dose-dependent manners These results were consistent with the previous studies, which indicated that sevoflurane significantly decreased the expression of MMP-9 in human adenocarcinoma A549 cells (Liang et al., 2013).

Induction of cancer cell apoptosis also is the main purpose in the treatment of cancers. In our research, we found that sevoflurane induces U2OS and Saos-2 cell apoptosis by decreasing the expression of anti-apoptotic protein, Bcl2, and increasing the expressions of pro-apoptotic proteins (cleaved caspase 3 and cleaved caspase 8). Considering Kvolik S et al., had proved that sevoflurane induces Caco-2 and HEp-2 cell apoptosis by inhibited the activation of PI3K/AKT signaling pathway (Lu et al., 2017), we can proposed that sevoflurane induces tumor cell apoptosis via regulating PI3K/AKT pathway.

The PI3K/AKT pathway is one of the most important oncogenic pathways in human cancer cells (Mao et al., 2013). Several evidences showed that PI3K/AKT signaling pathway was frequently hyperactivated in osteosarcoma cells and contributes to tumorigenesis, tumor cell proliferation, invasion, cell cycle progression, inhibition of apoptosis, angiogenesis, metastasis and chemo-resistance (Ye et al., 2012; Ye et al., 2015). Lu et al. investigated the in vivo and in vitro effects of miR-665 on sevoflurane anesthesia-induced cognitive dysfunction, and found that P13K/AKT signaling pathway was inactivated by sevoflurane (Lana et al., 2000). In our study, sevoflurane inhibited the activation of the PI3K/AKT pathway in dose-dependent and time-dependent manner in U2OS and Saos-2 cells. In addition, activator of PI3K/ATK pathway, IGF-1, obviously alleviated the sevoflurane-induced U2OS and Saos-2 cell proliferation and migration inhibition, as well as cell apoptosis, which further evidenced that sevoflurane exerted anticancer effects on osteosarcoma cells at least by inactivating PI3K/AKT pathway.

EMT is a importantly biological process in tumor metastasis which play a critical role in tumor cell migration and invasion (Acosta et al., 2011). This process decreases the expressions of cell-cell adhesion molecule, such as E-cadherin, and up-regulates the expressions of plastic mesenchymal proteins, including vimentin, N-cadherin and smooth muscle actin (Han and Yap, 2012; Jeanes et al., 2008). EMT process allows the tumor cells to gain increased migratory properties and invasiveness, which are critical steps in the process of tumor metastasis and leading to cancer spreading and treatment failure (Azmi, 2013; Lim and Thiery, 2012; Patel et al., 2003). In the present study, we discovered that sevoflurane decreased the expressions of N-cadherin and Fibronectin, and increased the expressions of E-cadherin and β-

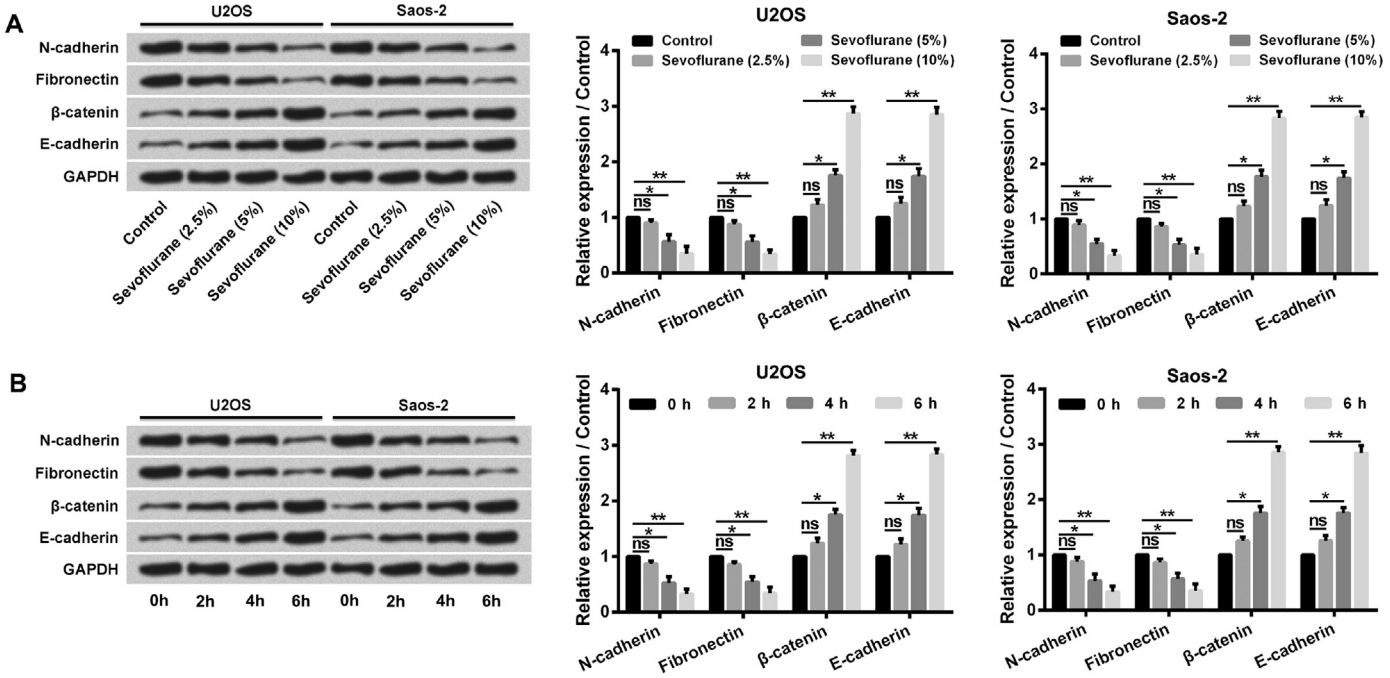

Fig. 6. Sevoflurane inhibits EMT process in osteosarcoma cells.
(A) Western blotting was used to measure the levels of epithelial markers (E-cadherin and β-catenin) and mesenchymal markers (Fibronectin and N-cadherin) following exposure to 2.5%, 5% or 10% sevoflurane treatment for 6 h. (B) Effects of 5% sevoflurane treatment for 2, 4, 6 h on the levels of epithelial markers (E-cadherin and β-catenin) and mesenchymal markers (fibronectin and N-cadherin) in U2OS and Saos-2 cells were analyzed by western blotting. EMT: Epithelial-mesenchymal transition. NS: No significant. $^{*}P < 0.05$, $^{**}P < 0.01$.

catenin in dose-dependent and time-dependent manner in U2OS and Saos-2 cells. These findings also suggested that sevoflurane inhibited EMT process in osteosarcoma cells.

Study has shown that surgical procedures may increase the invasion and migration potential of cancer cells and thus promote their ability to disseminate during the perioperative period (Coffey et al., 2003). Therefore, treatment with sevoflurane might play a vital role in reducing the tumor recurrence after surgery. Also, it is essential to choose proper anesthesia in process of tumor surgical resection. The anesthesia which has inhibitory properties against the proliferation, migration and invasion of cancer cells is best choice. Exploration of the molecular mechanisms which involved in the effects of anesthesia on osteosarcoma cell proliferation and migration may pave the path for the development of new approaches in the therapy of osteosarcoma. In conclusion, our findings suggest that sevoflurane inhibits the proliferation, migration and invasion of osteosarcoma cells by inactivating the PI3K/AKT pathway. However, considering that there are differences between in vitro exposure and in vivo exposure based on the differences between the percentages used in the experimental plan and the maximum allowable concentration (MAC) used in anesthesia, as well as the potential disparities in serum concentration. Further in vivo and clinical studies will be helpful for confirm the anti-tumor effect of sevoflurane on osteosarcoma, as well as the suitable concentration of sevoflurane in clinical use.

Conflict of interest

Authors declare that there is no conflict of interests.

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