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Sevoflurane induces apoptosis and inhibits the growth and motility of colon cancer in vitro and *in vivo* via inactivating Ras/Raf/MEK/ERK signaling

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ABSTRACT

Aims: To investigate the effects of sevoflurane on proliferation, cell cycle, apoptosis, autophagy, invasion and epithelial-mesenchymal transition of colon cancer cell line SW480, and to explore its possible mechanism.

Materials and methods: SW480 and SW620 cells were treated with a mixture of 95% O_2 + 5% CO_2 containing different concentrations of sevoflurane (1.7% SAV, 3.4% SAV and 5.1% SAV) for 6 h. Meanwhile, we performed a rescue experiment by treating cells with the ERK pathway activator LM22B-10 prior to treatment of cells with 5.1% sevoflurane。

Key findings: High concentration (5.1%) of sevoflurane significantly inhibited the proliferation and invasion of cells, causing G0/G1 phase arrest and promoted apoptosis and autophagy. 5.1% sevoflurane can participate in the regulation of EMT by regulating the expression of E-cadherin, Vimentin and N-cadherin proteins. LM22B-10 promoted proliferation and invasion of cancer cells and inhibited apoptosis and autophagy, while 5.1% sevoflurane could reverse the effect of LM22B-10 on the biological characteristics of cells. Sevoflurane can significantly inhibit tumor growth in SW480 cells transplanted nude mice. Moreover, 5.1% sevoflurane significantly increased the expression of p-Raf, p-MEK1/2, and p-ERK1/2 in SW480 cells and tumor tissues without affecting p-JNK and p-p38 proteins, meanwhile, 5.1% sevoflurane can inhibit the activation of ERK signaling pathway by LM22B-10 in vitro and *in vivo*.

Significance: Sevoflurane can inhibit the proliferation and invasion of colon cancer cells, induce apoptosis and autophagy, and participate in the regulation of epithelial-mesenchymal transition, which may be related to its inhibition of the ERK signaling pathway.

1. Introduction

Colon cancer is one of the most common malignant tumors in the world. There are about 600,000 new cases every year in the world [1], which poses a huge threat to human life and health. At present, the treatment of colon cancer is based on surgery, radiotherapy, chemotherapy and targeted therapy, but the clinical treatment effect is still not optimistic. Recurrence and metastasis of tumors are the main cause of poor prognosis in patients with colon cancer [2]. Exploring more effective treatments to improve patient outcomes is imminent.

Perioperative factors can affect the metastasis and recurrence of tumors, thus affecting the prognosis of patients [3]. Anesthesia, as an important component of surgery, plays an important role in postoperative outcomes. Some retrospective studies have found that inhaled anesthesia can improve the prognosis of patients with breast cancer, colon cancer, ovarian cancer and other cancer patients, and improve patient survival [4,5]. Previous studies have focused on the effects of anesthetics on patients' immunity and neuroendocrine [6,7], but little research has been done on the biological characteristics of tumors. It has been reported in the literature that the inhaled anesthetic drugs sevoflurane and isoflurane have inhibitory effects on the proliferation of various tumor cells, and are related to the time of anesthetic action [8]. As one of the commonly used inhaled anesthetics in clinical practice, sevoflurane is widely used in the maintenance of anesthesia in patients with colon cancer, but there are few studies on the biological characteristics of colon cancer cells. Kvolik et al. [8] simulated the effects of clinical use of sevoflurane on colon cancer SW620 cell line in vitro, and found that sevoflurane inhibited tumor cell growth and induced apoptosis in a time-dependent manner. However, the mechanism of apoptosis induction is still unclear.

The ERK signaling pathway is thought to be the classical mitogen-activated protein kinase (MAPK) signaling pathway. The classical pathway of the ERK signaling pathway is the Ras-Raf-MEK1/2-ERK1/2 pathway, which is composed mainly of a three-stage enzyme-linked

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functional unit, that is, Raf, MEK1/2, and ERK1/2 kinase are activated by phosphorylation in sequence [9,10]. First, Ras is converted to activated Ras by extracellular signal stimulation, and activated Ras phosphorylation activates Raf. Activation of Raf reactivates MEK1/2, and MEK1/2 phosphorylates to finally activate ERK1/2. Activated ERK1/2 eventually phosphorylates transcription factors in the cytoplasm and nucleus, regulates gene expression, and ultimately mediates cell growth, differentiation, migration, invasion and other processes. Abnormal activation of the Ras/Raf/MRK/ERK signaling pathway is closely associated with the development and malignant progression of a variety of malignancies, including colon cancer, and has been identified as a novel target in cancer therapy [11,12]. Research on Ras/Raf/MEK/ERK is a hot topic in current research [13]. The study of the role of Ras/Raf/MEK/ERK signaling pathway in colon cancer development may provide a potential theoretical basis for the development of new therapies for colon cancer.

In this study, we explored the effects of sevoflurane on the proliferation, apoptosis and invasion of colon cancercells and the possible molecular mechanisms of its effects, so as to explore the anti-tumor effect of sevoflurane. We hope to provide reference for the clinical application of anesthetics.

2. Materials and methods

2.1. Main reagents and instruments

Human normal colonic epithelial cells (NCM460), the human colon cancer cell line SW480 and SW620 was purchased from the Shanghai Institute of Cell Biomedicine, Chinese Academy of Sciences. Sevoflurane (Baxter, USA); RPMI1640, 10% FBS (Gibco, USA); MTT, Annexin V-FITC Apoptosis Detection Kit (Guo'an Biotechnology, China); Trypsin Cell Digestion (Biyuntian Biotechnology, China); flow cytometry, microplate reader (FACScan, BD Biosciences, USA); anesthetic gas monitor (Datex AS-3, Finland); Aestiva®/5 anesthesia machine (Datex Ohmeda, Finland); SEVOrane® type sevoflurane volatile tank (Abbott, USA).

2.2. Cell culture and drug treatment

After resuscitation, the cells were routinely cultured in RPMI1640 medium containing 10% fetal calf serum, and cultured at 37 °C under 5% CO_2 and saturated humidity. The cells in the logarithmic growth phase were prepared as single cell suspensions, seeded in 96-well culture plates (5×10^3/ml, 200 μl/well) and Transwell chambers (Corning, NY, USA), and the cells were routinely cultured until the cells were fully attached.

Sevoflurane treatment: Placed the plate in a sterile, closed container. A mixed gas containting 95% O_2 + 5% CO_2 containing sevoflurane was introduced from the inlet of the aseptic closed container, and the outlet port was connected to the Datex AS-3 anesthetic gas monitor to maintain the concentration of sevoflurane in the vessel at 1.7% (1.7% SAV group), 3.4% (3.4% SAV group), and 5.1% (5.1% SAV group). The aseptic closed container was placed in a 37 °C water bath for 6 h.

Ras/Raf/MEK/ERK signaling pathway activator treatment: LM22B-10 (Sigma-Aldrich, USA) was added to the plate to a final concentration of 50 μmol/L. After inoculation of SW480 cells, the plates were incubated for 1 h at 37 °C under 5% CO_2 and saturated humidity. Transfered the culture plate to a sterile closed container, passed a mixture of 95% O_2 + 5% CO_2 containing 5.1% sevoflurane (LM + SAV group), or only a mixture of 95% O2 + 5% CO_2 (LM group), continuous cultured for 6 h. After inoculation of SW480 cells, cultured at 37 °C under 5% CO_2 and saturated humidity for 1 h, the plate was placed in a sterile closed container, and a mixture of 95% O_2 + 5% CO_2 containing 5.1% sevoflurane (LM + SAV group), or only 95% O_2 + 5% CO_2 mixed gas (LM group) was introduced, continuous culture for 6 h.

The control group only passed a mixture of 95% O_2 + 5% CO_2. Seted nine duplicate holes in each group. After the treatment of each group was completed, the cells were returned to the 37 °C 5% CO_2 incubator for further 24 h, and subsequent experiments were carried out.

2.3. MTT assay for cell proliferation

After SW480, SW620 and NCM460 cells inoculated into 96-well plates were gas-treated and/or stimulated by drugs, they were transferred to a 37 °C 5% CO_2 incubator for 24 h. Cell proliferation was detected by MTT assay. In brief, 20 mL of MTT solution (5 mg/ml) was sequentially added to a 96-well plate, and after culturing for 6 h in an incubator, the cell culture medium was aspirated. 200 μL of DMSO was added to each well, and after incubation for 15 min in the dark, the absorbance was measured in a microplate reader (490 nm wavelength).

2.4. PI cytometry assay

The effect of sevoflurane on cell cycle was measured by flow cytometry (FACScan, BD Biosciences, USA). After the SW480 and SW620 cells were gas-treated and/or stimulated by the drug, the cells were further cultured for 24 h, and then the cells were resuspended in 70% ethanol at 4 °C overnight. Cells were stained with 500 μL PI/RNase Staining Buffer (BD Bioscience, USA) and analyzed for cell cycle using flow cytometry and FlowJo software.

2.5. Western blot assay

Western Blot assay was used to analyze the protein expression in cells and tissues. Total cellular protein was extracted using RIPA lysate. Protein concentration was determined by the BCA method (PIERCE, USA). The protein was separated by electrophoresis on 10% SDS-PAGE, transferred to a polyvinylidene fluoride membrane (Millipore, MA, USA), and blocked with 5% skim milk powder for 2 h at room temperature, and then washed by TBST. Membranes were incubated overnight at 4 °C with primary antibody to cyclin-E1, cyclin-D, Bcl-2, Bax, MMP-2, MMP-9, Vimentin, N-cadherin, and E-cadherin, Raf, p-Raf, MEK1/2, p-MEK1/2 , ERK1/2, p-ERK1/2, JNK, p-JNK, p38, p-p38 and internal control β-actin. After 5 washes of TBST, the membranes were incubated with HRP-conjugated secondary antibody for 1 h at room temperature, and after TBST washing, ECL chemiluminescence was developed. Relative protein expression levels were assessed using Quantity One software.

2.6. Annexin V/PI double staining assay

The effect of sevoflurane on apoptosis of SW480 and SW620 cells was detected by Annexin V/PI double staining. Cells were cultured for 48 h after gas treatment an/or drug stimulation. The cells were collected, digested with 0.25% trypsin, resuspended in 200 μL Binding buffer, and mixed with 0.5 μL Annexin V (KeyGen, Nanjing, China). Incubated for 5 min at room temperature in the dark, added 10 μL of PI, and incubated for 10 min in the dark. Apoptosis was detected by flow cytometry. The total percentage of apoptotic cells, annexin V-FITC positive, was demarcated as the sum of both early and late facets of apoptosis [14].

2.7. Analysis of caspase 3 activity

After the cells were ground in liquid nitrogen, they were suspended in the lysate. After centrifugation at 12,000 g for 5 min at 4 °C, the supernatant was collected for use. Protein concentration was determined using the method of Bradford [15]. The activity of Caspase-3 was measured by measuring the degree of fragmentation of the fluorescent substrate Ac-DEVD-pNA. The absorbance of the free fluorescent pNA was measured at 405 nm [16].

2.8. Immunofluorescence staining

Colon cancer cells were seeded in 24-well plates (5×10^4 cells/well) and cultured overnight. The corresponding concentration of drug was administered for 24 h according to the grouping. The medium was aspirated and the plate was washed with PBS buffer. After adding ice methanol to each well for 30 min, it was washed three times with pre-cooled PBS. 0.2% Triton X-100 was added to each well, and after 10 min of reaction, it was washed with PBS. Each well was blocked with 1 mL of blocking solution (5% BSA) for 1 h and washed with PBS. 200 μL of LC3 polyclonal antibody was added to each well overnight at 4 °C. After washing with PBS, 200 μL of the corresponding FITC-labeled fluorescent secondary antibody was added and incubated for 1 h in the dark. After washing with PBS, 200 μL of nuclear staining solution (DAPI) was added to each well to protect against light for 10 min at room temperature. After washing with PBS, the autophagy level of the cells was observed under a fluorescence microscope.

2.9. Transwell assay

The effect of sevoflurane on the invasion of SW480 and SW620 cells was detected by Transwell assay. Cells were cultured for 48 h after gas treatment and/or drug stimulation. Matrigel gel (Corning, NY, USA) was allowed to melt overnight at 4 °C. Matrigel gel was placed on ice and diluted 8 times with a pre-cooled pipette into a cold serum-free medium, and 50 μL was added to the upper chamber of a Transwell chamber. The cells were seeded at 2×10^5 cells/mL into the upper chamber of the Transwell chamber, and 500 μL of a culture solution containing 20% fetal calf serum was added to the lower chamber, and cultured at 37 °C under 5% CO_2 for 24 h. Discarded the upper and lower culture medium, wiped off the upper chamber for more liquid, placed in 10% formalin for 1 h at room temperature, stained with crystal violet for 20 min, and photographed and counted under a 200 × microscope. The number of transmembrane cells was recorded by selecting 10 fields per membrane.

2.10. Tumor xenograft growth assay in vivo

BALB/c female nude mice (4–6 weeks old) weighing 18–20 g were purchased from Antaik Biotechnology Co., Ltd (Beijing, China), and reared under SPF conditions. SW480 cells were cultured for 24 h after gas treatment and/or drug stimulation. Then, SW480 cells were digested with 0.25% trypsin-0.2% EDTA, and then centrifuged to prepare a 2×10^6 cells/mL single cell suspension, which was inoculated subcutaneously into the right anterior temporal region of nude mice. Each mouse was injected with 0.2 mL, and 5 mice per group were set. When the tumor mass of the underarm of the nude mouse was visible to the naked eye, the longest diameter (a) and the shortest diameter (b) of the transplanted tumor were measured every 3 days using an electronic digital caliper (Guanlu, China), and the tumor volume (V) was calculated according to the formula: $V = 1/2ab^2$ (mm^3). On the 21st day, the mice were sacrificed by cervical dislocation, and the right anterior subcutaneous tumor of nude mice was completely stripped, and the tumor weight was weighed.

2.11. Immunohistochemical analysis

Fresh tumor tissue specimens were fixed in 10% neutral formalin solution, dehydrated with gradient alcohol, and toluene was transparent to prepare paraffin sections (5 μm). The sections were dewaxed by xylene and hydrated by gradient alcohol. Antigen retrieval was carried out in citrate buffer at 95 °C for 15 min, and peroxidase blocker was added for 30 min. After washing with PBS, the cells were incubated with normal non-immune serum for 30 min, serum was removed, Ki67 antibody was added dropwise to the slide, and incubation was carried out at 37 °C for 1 h. Sections incubated with PBS served as negative controls. The biotinylated secondary antibody was incubated on the slide for 30 min, and incubated with streptomycin avidin-peroxidase for 15 min. After washing with PBS, the DAB solution was added for color development. Observed under a light microscope: brown or brownish yellow particles appearing inside the cells are positively stained cells.

2.12. Statistical analysis

Data were expressed as means ± standard deviation (SD) and analyzed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Statistical difference between groups was analyzed by unpaired *t*-test. One-way analysis of variance was used for multiple comparison. Results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effects of sevoflurane on proliferation and cell cycle of colon cancer cells

Malignant proliferation is an important biological property of colon cancer cells. In this study, we selected the concentration and duration of sevoflurane commonly used in clinical trials, that is, 1.70%, 3.4%, and 5.1% sevoflurane (equivalent to 1, 2, 3 MAC in clinical trials) [17] were applied to NCM460, SW480 and SW620 cells for 6 h, then transferred to the normal environment for 48 h, and analyzed cell proliferation by MTT assay. We found that different concentrations of sevoflurane did not affect the proliferation of human normal colonic epithelial cells NCM460. However, compared with the control group, the OD_{450} value of SW480 and SW620 cells in 1.7%SAV, 3.4%SAV and 5.1%SAV group was significantly reduced ($p < 0.05$, Fig. 1A).

The malignant proliferation of tumors is closely related to abnormal cell cycle progression. To analyze the reasons why sevoflurane inhibited the proliferation of colon cancer cell line SW480 and SW620, we examined the distribution of cell cycle. We treated SW480 cells with different concentrations of sevoflurane for 6 h, then transferred to the conventional culture environment for 24 h, and the cell cycle distribution was detected by PI single staining assay. We found that, compared with the control group, the proportion of G0/G1 phase cells in 1.7%SAV, 3.4%SAV and 5.1%SAV group was significantly increased, and the proportion of cells in the S phase was significantly decreased ($p < 0.05$), while the difference in the proportion of cells in the G2/M phase was not statistically significant (Fig. 1B).

We further examined the expression of cyclin-D1 and cyclin-E, which are involved in the regulation of the transition between G0/G1 and S phases. We found that 1.7%, 3.4% and 5.1% sevoflurane significantly inhibited the expression level of cyclin-D1 and cyclin-E in SW480 and SW620 cells compared to the control group ($p < 0.05$, Fig. 1C). The results suggested that sevoflurane can inhibit the proliferation of SW480 cells by regulating the expression of cell cycle-associated proteins, causing G0/G1 arrest in SW480 and SW620 cell cycle.

3.2. Effects of sevoflurane on apoptosis and autophagy of colon cancer cells

Apoptosis is a programmed and active cell death regulated by genes, which is closely related to tumor progression and malignant biological behavior. Next, we used flow cytometry to analyze the effect of sevoflurane on apoptosis of SW480 and SW620 cells. We cultured SW480 and SW620 in an environment containing a mixture of different concentrations of sevoflurane for 6 h, and then transferred to a normal environment for 48 h. As shown in Fig. 2A, compared with the control group, the apoptotic rate in 1.7%SAV, 3.4%SAV and 5.1%SAV group was significantly increased ($p < 0.05$). Bcl-2 and Bax are key regulators of apoptosis. Compared with the control group, 1.7%SAV, 3.4% SAV and 5.1% sevoflurane significantly increased Bax protein expression and inhibited Bcl-2 protein expression ($p < 0.05$, Fig. 2B).

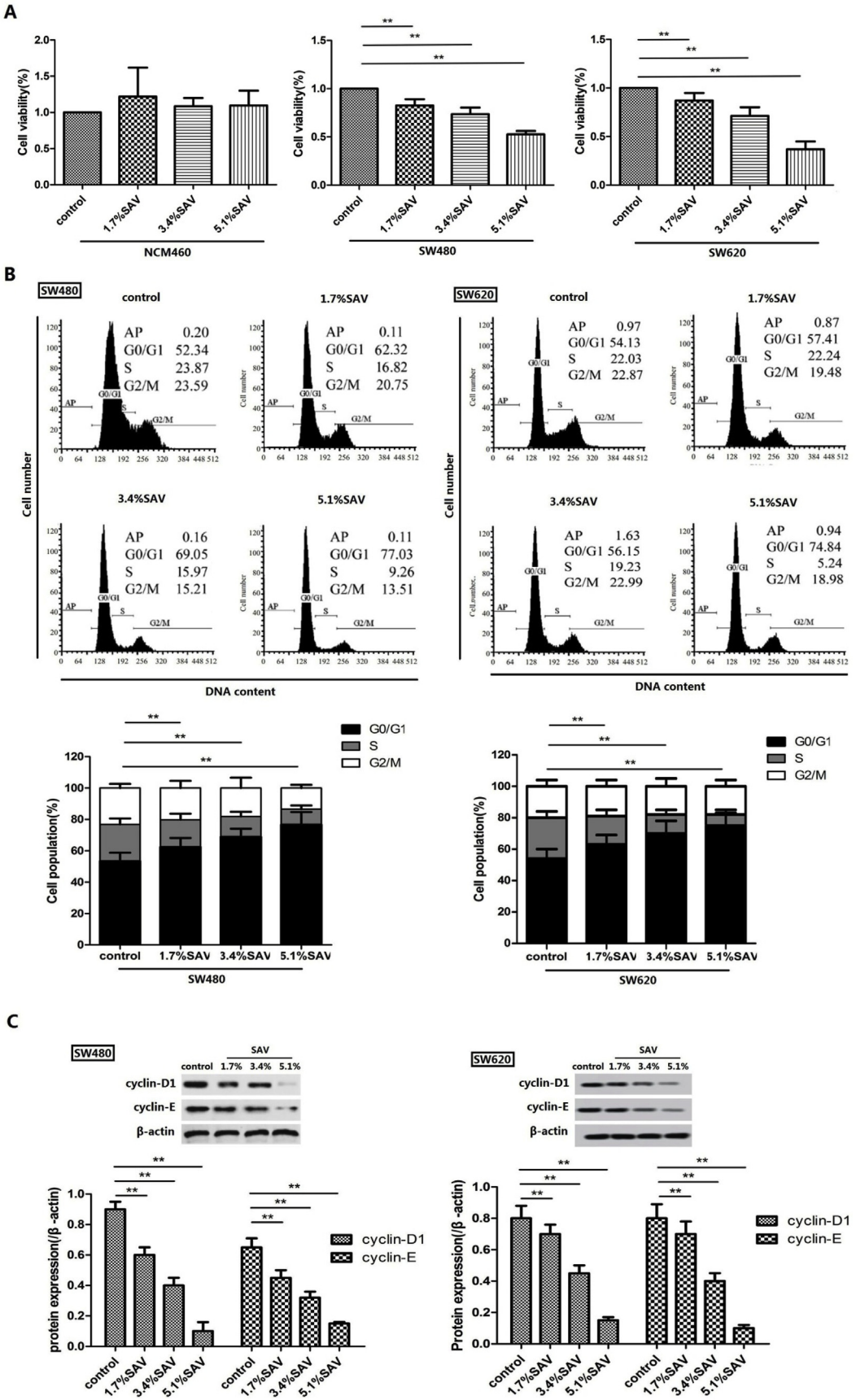

Fig. 1. Effect of sevoflurane on proliferation and cell cycle progression of SW480 cells. A: MTT assay showed that 1.7%, 3.4% and 5.1% sevoflurane significantly inhibited the proliferation of SW480 cells. B: The proportion of cells in the G0/G1, S and G2/M phases were analyzed by PI staining and flow cytometry. C: The effect of sevoflurane on the expression of Cyclin-D1 and cyclin-E in the downstream cell cycle was analyzed by western blotting. *$p < 0.05$, **$p < 0.01$.

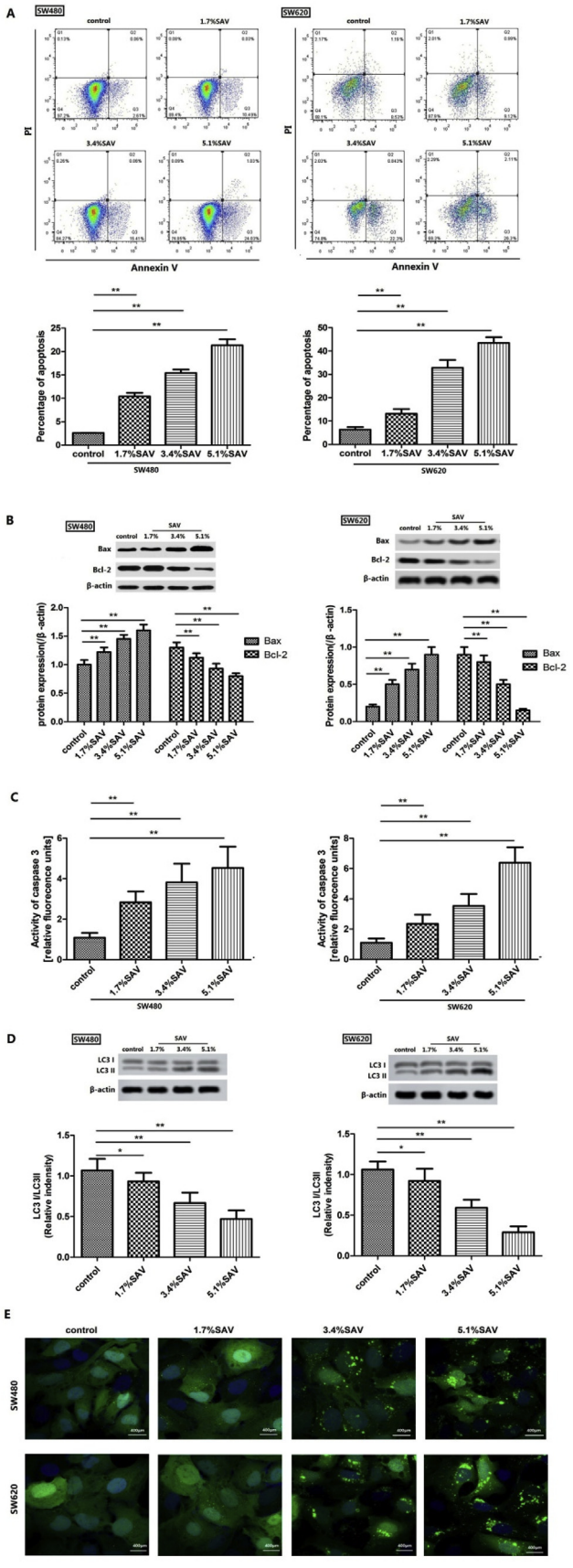

Fig. 2. Effect of sevoflurane on apoptosis of SW480 cells. A: The effect of sevoflurane on apoptosis in SW480 cells was determined by flow cytometry with Annexin V/PI double staining. B: The expression of downstream marker proteins in apoptotic pathway, Bcl-2 and Bax, in SW480 and SW620 cells was detected by western blotting. C: The activity of caspase-3 in SW480 and SW620 cells. D: The expression of autophagy marker proteins Beclin-1 and LC3 was detected by Western blot assay. E: The effect of Sevoflurnane on autophagy in SW480 and SW620 cells was analyzed by immunofluorescence staining. Blue: DAPI-labeled nuclei; Green: Immunofluorescently labeled LC3 protein. Scale bar = 400μm $*p < 0.05$, $**p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Additionally, sevoflurane also can significantly increase the activity of caspase 3 in SW480 and SW620 cells (Fig. 2C). The results revealed that sevoflurane can regulate the expression of apoptosis-related proteins and induce apoptosis of SW480 and SW620.

Autophagy plays an important role in cell waste removal, structural remodeling, and growth. LC3 is the characteristic protein of the autophagy process [18]. The decrease in the proportion of LC3 I/LC3 II in cellular proteins represents the occurrence of autophagy [19]. We first used Western blot assay to analyze the content of LC3 I and LC3 II in cells to evaluate the effect of sevoflurane on autophagy of colon cancer cells. We found that as the concentration of sevoflurane increased, the expression of LC3 II increased, while the proportion of LC3 I/LC3 II decreased significantly ($p < 0.05$, Fig. 2D). We also observed the effect of sevoflurane on autophagy by immunofluorescence staining (Fig. 2E). The cytoplasmic LC3 fluorescence (green) of colon cancer cells in the control group was less and showed a diffuse state. In contrast, after treatment with sevoflurane for 24 h, the LC3 green fluorescence in the colon cancer cells was relatively concentrated and the fluorescent spots increased. These results indicated that the degree of autophagy in colon cancer cells is positively correlated with drug concentration.

3.3. Effects of sevoflurane on invasion and epithelial-mesenchymal transition (EMT) in colon cancer cells

Metastasis is the leading cause of death in colon cancer patients, and cell invasion is a hallmark of cancer metastasis. We next analyzed the effect of sevoflurane on the invasion of SW480 and SW620 cells by transwell assay. We found that the number of invading cells in 1.7% SAV, 3.4%SAV and 5.1%SAV group was significantly lower than that of the control group after sevoflurane was applied to SW480 and SW620 for 6 h (Fig. 3A). Matrix metalloproteinase-9 (MMP-9) and MMP-2 are the most important marker proteins in tumor metastasis and invasion. We also found that sevoflurane significantly inhibited MMP-9 and MMP-2 protein expression in SW480 and SW620 cells (Fig. 3B).

As an invasive phenotype of cancer cells, EMT plays an important role in tumor metastasis. During the EMT process, the expression of the interstitial marker proteins Vimentin and N-cadherin increased, and the expression of the epithelial marker protein E-cadherin decreased. Therefore, EMT can be evaluated by measuring the expression levels of Vimentin, N-cadherin, and E-cadherin in cells [20]. Western blot assay showed that the expression level of E-cadherin was significantly increased in SW480 and SW620 cells in 1.7%SAV, 3.4%SAV and 5.1% SAV group, and the expression levels of Vimentin and N-cadherin protein were significantly decreased compared to the control group ($p < 0.05$, Fig. 3C). The results suggested that sevoflurane can inhibit the invasion of colon cancer cell line SW480 and SW620 cells by regulating EMT.

3.4. Effect of sevoflurane on MAPK signaling pathway

The mitogen-activated protein kinase (MAPK) signaling pathway is the main signaling pathway involved in cell proliferation, differentiation, and apoptosis, and its cascade pathway plays an important role in

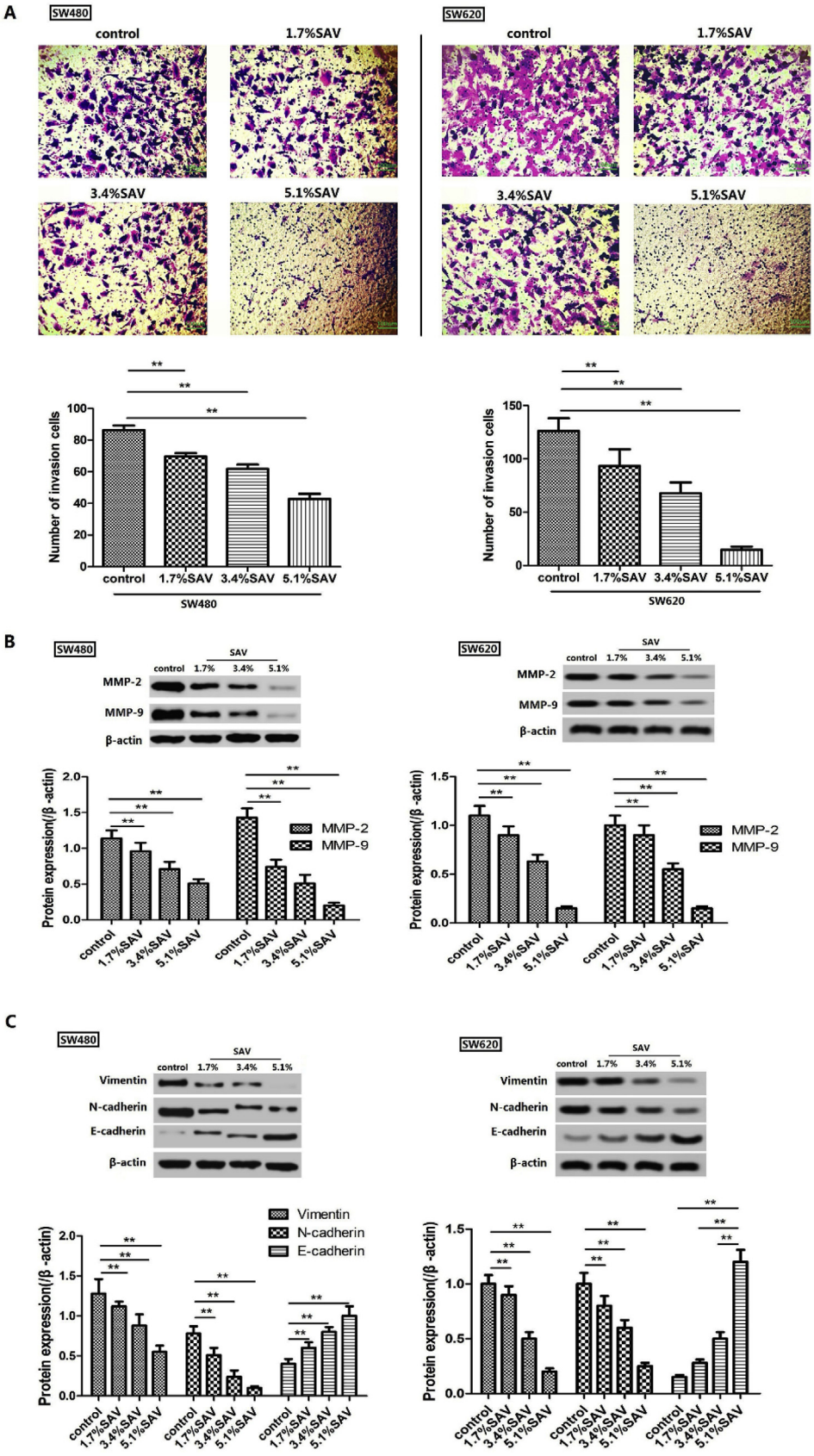

Fig. 3. Effect of sevoflurane on invasion and epithelial-mesenchymal transition of SW480 cells. A: Transwell assay showed that 1.7%, 3.4% and 5.1% sevoflurane significantly inhibited the invasion ability of SW480 cells. Scale bar = 100μm. B: The expression of downstream marker proteins in cell invasion-related proteins, MMP-2 and MMP-9, in SW480 cells was detected by western blotting. C: The expression of EMT marker proteins, Vimentin, N-cadherin and E-cadherin, in SW480 cells was detected by western blotting. *p < 0.05, **p < 0.01.

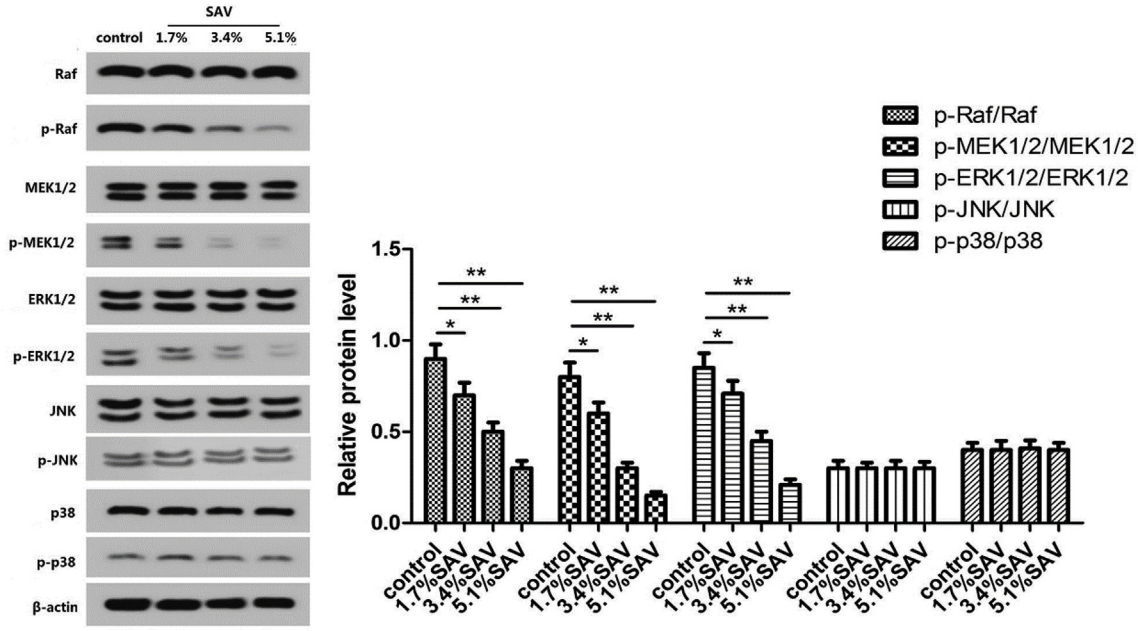

Fig. 4. Effect of sevoflurane on ERK signaling pathway. The expression of MAPK signaling pathway proteins in SW480 cells was detected by western blotting. *p < 0.05, **p < 0.01.

signal transduction. To investigate the mechanism of the effects of sevoflurane on the biological characteristics of colon cancer cell line SW480, we evaluated the activity of the MAPK signaling pathway in SW480 cells (Fig. 4). Compared with the control group, the levels of p-Raf/Raf, p-MEK1/2/MEK1/2 and p-ERK1/2/ERK1/2 in 1.7%SAV, 3.4% SAV and 5.1%SAV group were significantly decreased ($p < 0.05$), while the levels of p-JNK/JNK and p-p38/p38 were not significantly changed ($p > 0.05$), suggesting that sevoflurane may participate in its effects on the biological characteristics of SW480 cells by inhibiting phosphorylation of the ERK pathway, rather than the JNK and p38 pathways.

3.5. Sevoflurane can reverse the effects of ERK signaling pathway activator LM22B-10 on biological characteristics of colon cancer cells

To further confirmed whether sevoflurane can regulate the proliferation, apoptosis and invasion of SW480 cells by inhibiting the activation of ERK pathway, we performed a rescue experiment by treating cells with the ERK pathway activator LM22B-10 prior to treatment of cells with sevoflurane. As shown in Fig. 5A, the levels of p-Raf/Raf, p-MEK1/2/MEK1/2 and p-ERK1/2/ERK1/2 in the LM group were significantly higher than those in the control group, while the expression levels of Raf, MEK1/2 and ERK1/2 were not significantly changed, confirming LM22B-10 can phosphorylate the ERK signaling pathway protein, thereby activating the ERK signaling pathway. The levels of p-Raf/Raf, p-MEK1/2 /MEK1/2and p-ERK1/2/ERK1/2 in LM + SAV group were significantly lower than those in LM group and control group, suggesting that high concentration of sevoflurane treatment can significantly inhibit the activation of ERK signaling pathway by LM22B-10.

Further analysis of the biological characteristics of SW480 cells (Fig. 5B–E) showed that compared with the LM group, the proliferation and invasion ability of the cells in the LM + SAV group were significantly inhibited, and the apoptosis rate was significantly increased. In addition, compared with LM group, the expression levels of cyclin-D1, cyclin-E, Bcl-2, MMP-9, MMP-2, Vimentin and N-cadherin in LM + SAV group were significantly decreased, while E-cadherin and Bax were increased significantly ($p < 0.05$, Fig. 5F). Additionally, sevoflurane also inhibited LM22B-10-induced activation of caspase 3 (Fig. 5G). Moreover, sevoflurane reversed the inhibitory effect of LM22B-10 on autophagy (Fig. 5H and I). These results revealed that sevoflurane is involved in the regulation of proliferation, apoptosis, autophagy, invasion and EMT of SW480 cells by inhibiting the activation of ERK signaling pathway.

3.6. Sevoflurane can inhibit the tumorigenic ability of SW480 cells in vivo

Next, we used the tumor xenograft growth assay to study the inhibitory effect of sevoflurane on the tumorigenic ability of colon cancer cell SW480 *in vivo*. SW480 cells after gas treatment and drug stimulation were subcutaneously injected into the right anterior tibia of nude mice. As shown in Fig. 6A–C, the volume and weight of xenograft tumors in the LM group were significantly increased compared to the control group, while the SAV group was significantly reduced ($p < 0.05$). In addition, the volume and weight of xenograft tumors in the LM + SAV group were significantly lower than those in the LM group. The results revealed that SAV inhibits the growth of xenografts in nude mice, and this effect might be related to its inhibition of ERK signaling pathway activation.

Immunohistochemical staining analysis showed that the number of Ki-67 staining positive tumor cells on the tumor histopathological sections of nude mice inoculated with 5.1% sevoflurane-treated SW480 cells was significantly lower than that of the control group ($p < 0.05$). In contrast, the number of Ki-67 staining-positive tumor cells in nude mice inoculated with LM22B-10 treated SW480 cells was significantly increased ($p < 0.05$). Meanwhile, the number of Ki-67 staining-positive tumor cells in LM + SAV group was significantly lower than that of the LM group ($p < 0.05$, Fig. 6D), suggesting that sevoflurane inhibits proliferation of colon cancer cells *in vivo*.

We also examined the expression of cell cycle, apoptosis, and metastasis-associated proteins by western blotting. The results showed that the expression levels of cyclin-D1, cyclin-E, Bcl-2, MMP-9, MMP-2, Vimentin and N-cadherin in the transplanted tumor tissues of LM + SAV group and SAV group were significantly decreased compared to the control group, while E-cadherin, Bax expression levels increased significantly ($p < 0.05$, Fig. 6E), which was consistent with in vitro results. Additionally, the activity of caspase 3 (Fig. 6F) and the level of LC3 I/LC3 II (Fig. 6G) in LM + SAV group was significally

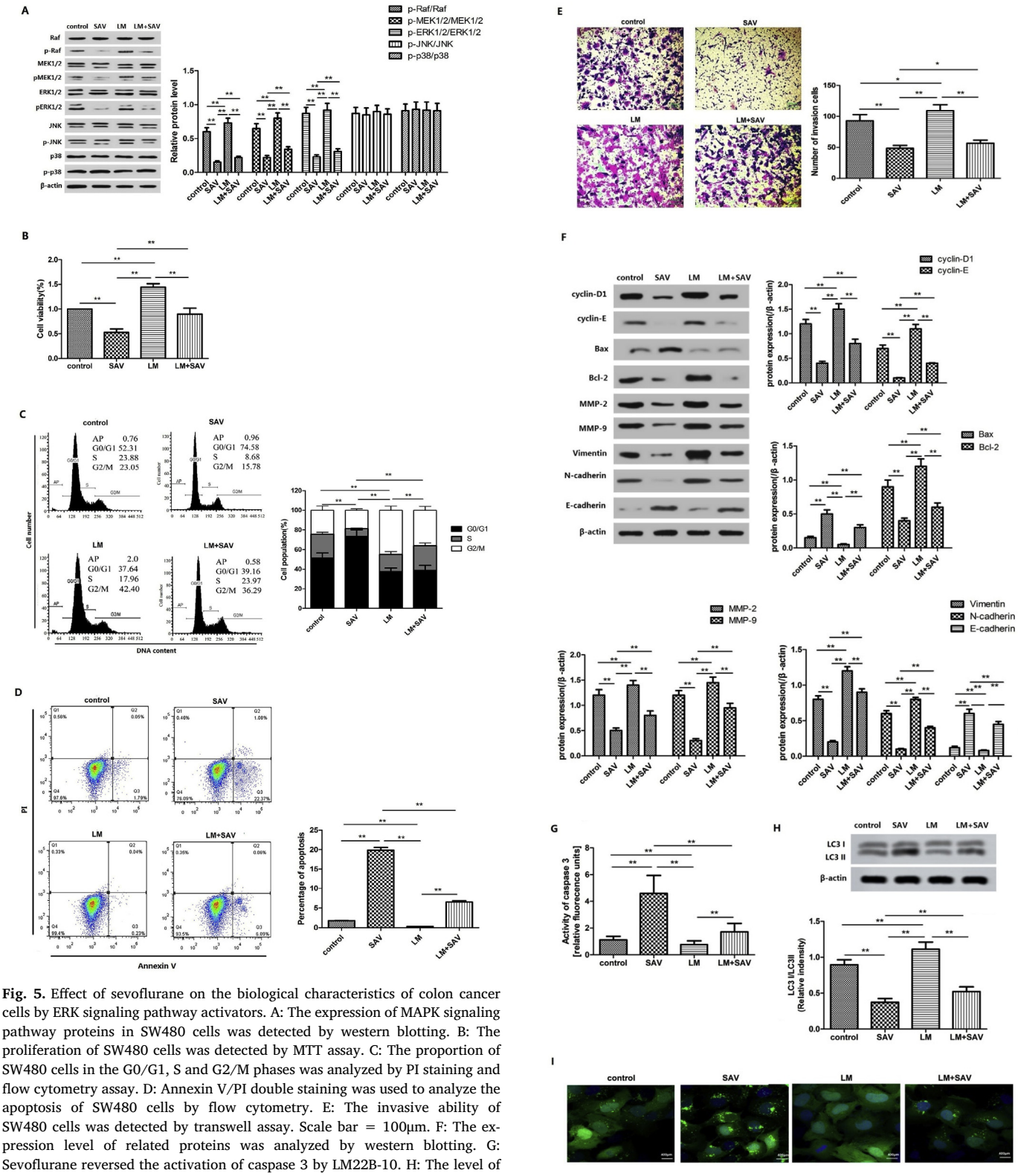

Fig. 5. Effect of sevoflurane on the biological characteristics of colon cancer cells by ERK signaling pathway activators. A: The expression of MAPK signaling pathway proteins in SW480 cells was detected by western blotting. B: The proliferation of SW480 cells was detected by MTT assay. C: The proportion of SW480 cells in the G0/G1, S and G2/M phases was analyzed by PI staining and flow cytometry assay. D: Annexin V/PI double staining was used to analyze the apoptosis of SW480 cells by flow cytometry. E: The invasive ability of SW480 cells was detected by transwell assay. Scale bar = 100μm. F: The expression level of related proteins was analyzed by western blotting. G: Sevoflurane reversed the activation of caspase 3 by LM22B-10. H: The level of LC3 I/LC3 II in SW480 cells was analyzed by Western blot assay. I: The autophagy of SW480 and SW620 cells was analyzed by immunofluorescence staining. Blue: DAPI-labeled nuclei; Green: Immunofluorescently labeled LC3 protein. Scale bar = 400μm *$p < 0.05$, **$p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 5. (*continued*)

lower than that of LM group ($p < 0.05$). In addition, Western blot assay showed that (Fig. 6H), the levels of p-Raf/Raf, p-MEK1/2/MEK1/2, and p-ERK1/2/ERK1/2 in LM + SAV group were significantly lower than those in LM group and control group, confirming that LM22b-10 can significantly activate the ERK signaling pathway, while sevoflurane can inhibit the activation of ERK signaling pathway by LM22B-10.

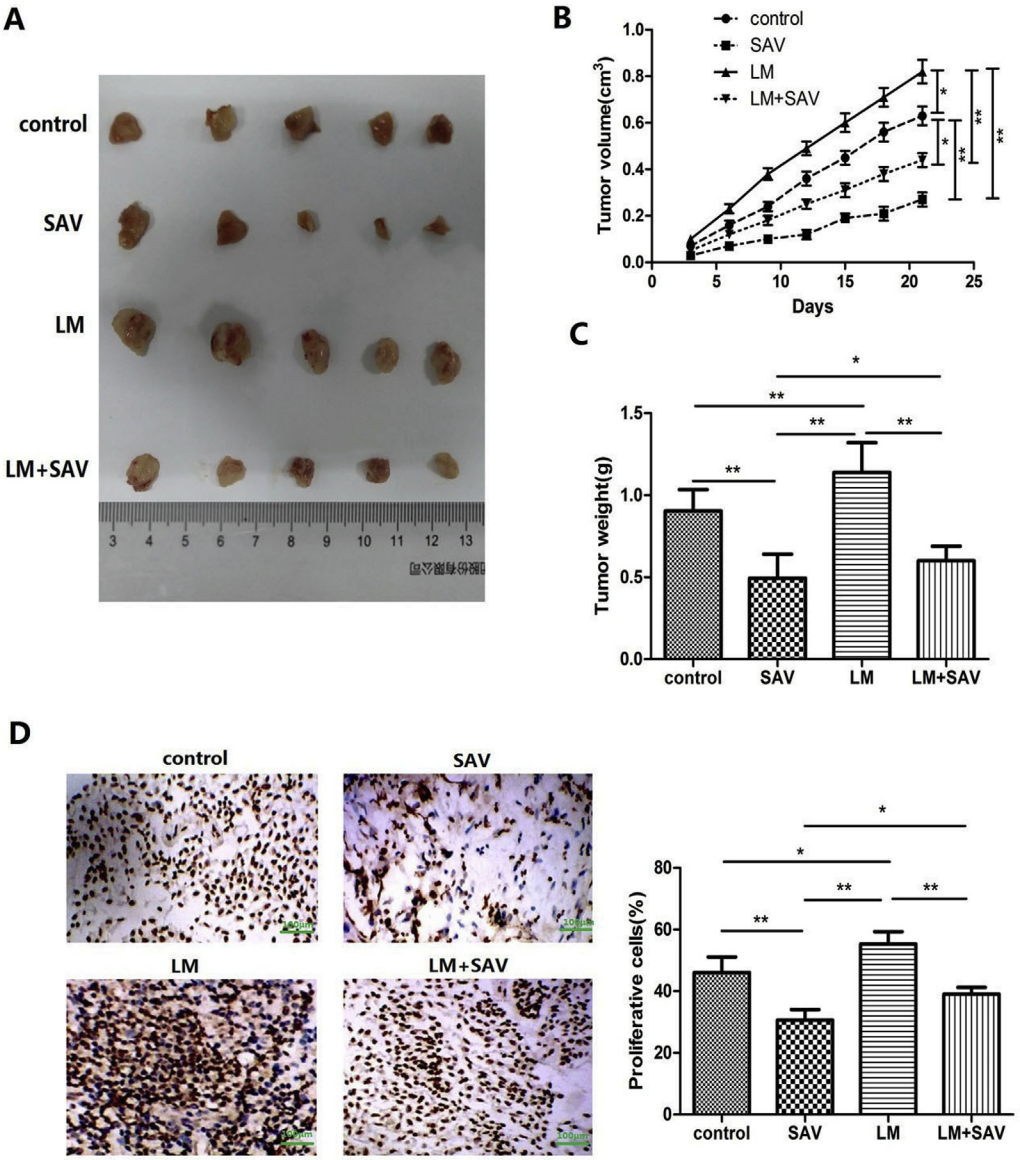

Fig. 6. Effect of sevoflurane on the subcutaneous tumorigenic ability of SW480 cells. A: Representative tumorigenic pictures. B: Statistical chart of tumor volume. C: Statistical chart of tumor weight. D: Ki-67 protein expression in tumor tissues was analyzed by immunohistochemical analysis. Scale bar = 50μm. E: The expression level of cell cycle, invasion, apoptosis and EMT-related proteins was detected by western blotting analysis. F: Sevoflurane inhibited the activity of caspase 3 in tumor tissues. G: The level of LC3 I/LC3 II in tumor tissues was analyzed by Western blot assay. H: The expression of MAPK signaling pathway proteins in SW480 cells was detected by western blotting. n = 5.*p < 0.05, **p < 0.01.

4. Discussion

Anesthetics and anesthesia methods can promote or inhibit the migration of tumor cells, which affects the long-term prognosis of cancer patients [21]. Sevoflurane is a commonly used inhaled anesthetic during colon cancer surgery and is widely used in the maintenance of intraoperative anesthesia in cancer patients. Recent studies have found that sevoflurane has potential anticancer effects on a variety of tumor cells. Kvolik et al. found that sevoflurane inhibited the growth of laryngeal carcinoma cell line HEP-2, but had no significant effect on the growth of Paca-2 and WI-38 cells [22]. Mitsuhata et al. [23] showed that sevoflurane can significantly reduce the release of IL-1 and TNF-α by inhibiting the release of cytokines produced by tumor stimulation by NK cells and NK-like cells, but has no effect on IL-2. G. Brozovic et al. [24] found that sevoflurane has a synergistic effect on cisplatin-induced genotoxicity mainly in peripheral blood leukocytes, hepatocytes, and kidney cells; while in neuronal and tumor cells, sevoflurane was found to antagonize cisplatin-induced genotoxicity. Huitink et al. [25] found that inhaled anesthetics (sevoflurane, isoflurane, etc.) can change the expression of apoptosis-related genes in neuroblastoma (SH-SY5Y) and breast cancer (MCF7) cells, thereby exerting anticancer effects. Recently, S. Kvolik et al. [8] found that sevoflurane can increase the apoptotic rate of colon cancer Caco-2 cells after treatment for 2 h, but the specific mechanism of action is not clear. Therefore, this study focused on the effects of sevoflurane on the biological characteristics of colon cancer cell line and its related mechanisms.

Malignant proliferation is an important biological characteristic of colon cancer cells, and tumor proliferation abnormalities are closely related to abnormal cell cycle progression. Abnormal cell cycle G1/S and G2/M phase transitions lead to cell cycle disorders, uncontrolled cell proliferation, and eventually canceration [26]. It has been reported that sevoflurane can regulate the proliferation and apoptosis of colon cancer cells [8], but the mode of regulation is still unclear. We found that sevoflurane inhibited the proliferation of SW480 and SW620 cells (Fig. 1A), resulting in a significant accumulation of tumor cells in the G0/G1 phase accompanied by a decrease in S phase cells (Fig. 1B) and

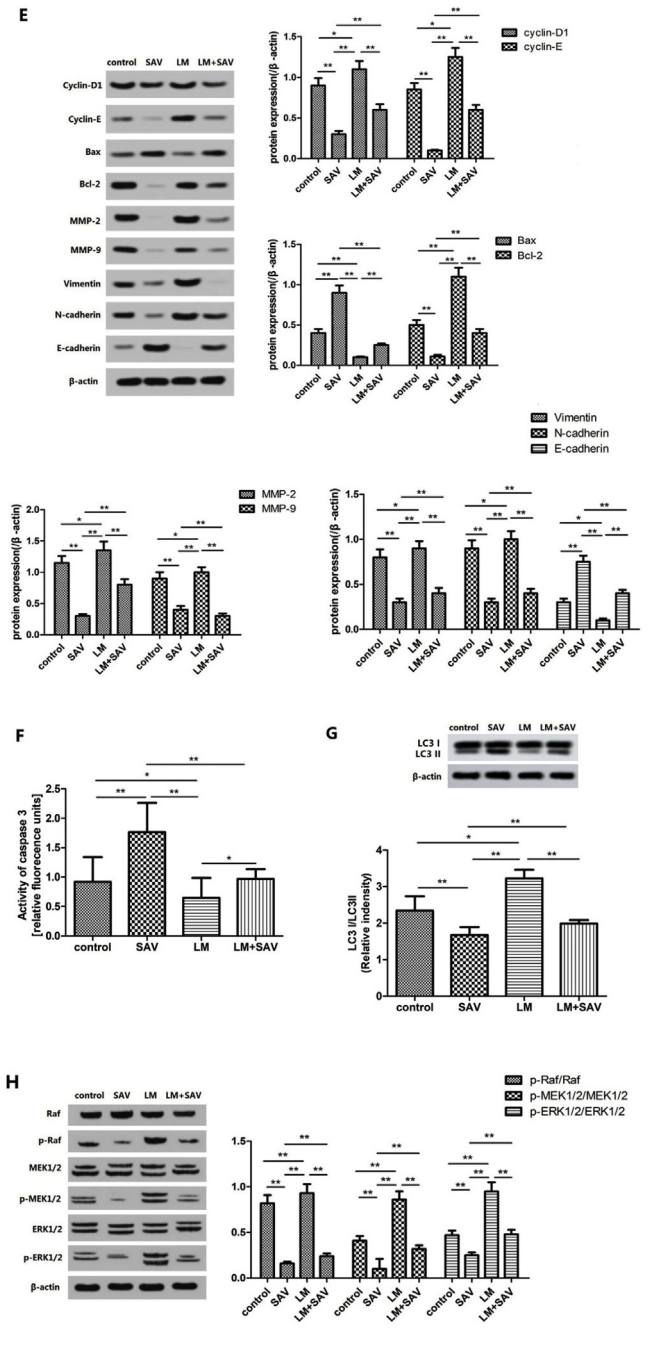

Fig. 6. (*continued*)

an increase in apoptosis (Fig. 2A). Studies have confirmed that the degradation of Cyclin D1 can cause G0/G1 phase arrest in the tumor cell cycle [27]. We also found that sevoflurane inhibited the expression of Cyclin D1 and Cyclin E proteins (Fig. 1C). Therefore, it is speculated that sevoflurane can inhibit the transduction of SW480 and SW620 cells from G0/G1 phase to S phase, and the cells are arrested in G0/G1 phase, resulting in decreased proliferation of colon cancer cells. Bcl-2 and Bax are key regulators of apoptosis. As the most important anti-apoptotic gene in anti-apoptotic family members, Bcl-2 can exert anti-apoptotic effects by binding to Bax to form isomeric dimers or regulating intracellular Ca^{2+} concentration [28]. Studies have shown that downregulation of Bcl-2 expression can induce apoptosis [29]. In addition, overexpression of the Bcl-2 family is also an important factor in tumor cell proliferation [30]. Our study also showed that sevoflurane significantly decreased Bcl-2 expression and increased Bax expression in SW480 and SW620 cells (Fig. 2B). Additionally, sevoflurane also improve the activation of caspase 3 in colon cancer cells (Fig. 2C), confirming that sevoflurane induced apoptosis in colon cancer cells.

Autophagy is a protective defense mechanism that exists in eukaryotes. Abnormal autophagy activity can lead to a variety of diseases including malignant tumors [31]. Treatment of tumors by regulating autophagy activity may become a new target in the field of tumor therapy. LC3 is the hallmark protein for autophagy [32]. When autophagy occurs, LC3 I in the cytoplasm is converted to LC3 II by phosphatidylethanolamine, which binds to the autophagosome membrane. Therefore, the level of LC3 I/LC3 II in cells can reflect the autophagy of cells [19]. We found that the proportion of LC3 I/LC3 II in colon cancer cell lines gradually decreased with increasing sevoflurane concentration(Fig. 2D). Moreover, immunofluorescence staining also showed that sevoflurane increased the amount of LC3 protein in the cells (Fig. 2E), suggesting an increase in the level of autophagy in colon cancer cells. These results revealed that sevoflurane can induce autophagy and inhibit the growth of colon cancer cells.

Metastasis is the leading cause of death in colon cancer patients. Migration and invasion are another important malignant biological behavior of colon cancer cells and a hallmark of cancer metastasis. Matrix metalloproteinase (MMPs)-induced degradation of extracellular matrix is a hallmark of cell invasion. It has been demonstrated that the invasive ability of cancer cells can be regulated by MMPs [33]. MMP-2 and MMP-9 are closely associated with increased invasion and metastasis in the advanced stage of the tumor [34]. We found that sevoflurane can significantly reduce the expression levels of MMP-2 and MMP-9 proteins, suggesting the inhibitory effect of sevoflurane on the invasion of colon cancer cells SW480 and SW620 (Fig. 3B). EMT is an invasive phenotype of cancer cells and a key event in the ability of colon cancer cells to acquire metastases [35]. Studies have shown that once colon cancer cells develop EMT, their ability to invade and metastasize is significantly enhanced [36]. The EMT process is characterized by the fact that cells of the epithelial phenotype continue to transform into interstitial phenotype cells, which in turn cause a decrease in intercellular polarity and adhesion mediated by the epithelial phenotype. After obtaining the interstitial phenotype, the cells easily leave the primary lesion and migrate to adjacent tissues [37]. EMT usually shows a decrease in the expression of the epithelial marker E-cadherin, an increase in the expression of the interstitial markers N-cadherin and Vimentin, and changes in the expression of these proteins impair cell adhesion, thereby promoting the spread of cancer cells [38,39]. We found that sevoflurane increased the expression of E-cadherin in SW480 and SW620 cells and decreased the expression levels of N-cadherin and Vimentin (Fig. 3C). It is suggested that sevoflurane can inhibit the invasion of colon cancer cells by regulating the expression of EMT marker gene to inhibit the EMT process (Fig. 3).

The mitogen-activated protein kinase (MAPK) signaling pathway is the main signaling pathway involved in cell proliferation, differentiation, and apoptosis, and its cascade pathway plays an important role in signal transduction. The MAPK signaling pathway mainly includes extracellular signal-regulated protein kinase (ERK), p38 pathway and c-Jun amino terminal kinase (JNK). Activation of the MAPK signaling pathway has been shown to be closely associated with the development of multiple tumors including colon cancer. We found that sevoflurane down-regulates phosphorylation of ERK signaling pathway-associated proteins without affecting phosphorylation levels of JNK and p38 signaling pathways (Fig. 4). Therefore, we speculated that the effect of sevoflurane on the biological characteristics of colon cancer cell SW480 may be related to its inhibition of ERK signaling pathway. To confirm this hypothesis, we pretreated SW480 cells with the ERK signaling pathway activator LM22B-10 for rescue experiments (Fig. 5). ERK signaling pathway is closely related to cell fate. Phosphorylation of ERK promotes cell proliferation by promoting protein synthesis, cyclin/cyclin-dependent kinase (cyclin/CDK) complex formation, and increased protein stability. The expression of cyclinD1 induced by the ERK

pathway is an essential protein for G1/S phase transition in the cell cycle [40]. Over-activated ERK signaling pathway blocks cell cycle progression and accumulates intracellular cyclinD1. Over-accumulated cyclinD1 binds to the cell cycle inhibitor p21cip1, causing cells to enter a resting state. We found that LM22B-10 promotes cell proliferation and accelerates cell cycle progression, while sevoflurane reverses the effects of LM22B-10. We hypothesized that the inhibitory effect of sevoflurane on the abnormal activation of the ERK pathway may result in a decrease in cyclin D1, leading to inhibition of cyclin/CDK complex synthesis to cause cell cycle arrest and ultimately inhibition of cell proliferation. Von et al. [41]conformed that Raf can inhibit apoptosis by translocation to mitochondria and inactivating Bad proteins by phosphorylation of residue S112. Furthermore, ERK can induce the activation of multiple transcription factors including nuclear factor (NF)-κB [42], and these transcription factors can further regulate the expression of cell cycle and apoptosis-related genes, such as CDKs and Bcl-2 , Bcl-XL, Fas-L and caspases [43]. This study also showed that the activation of ERK pathway inhibits apoptosis by promoting Bcl-2 expression while inhibiting BBax expression, while sevoflurane reverse the anti-apoptotic effect of LM22B-10 on colon cancer cells. Chantal et al. [44] found that PARP-1 induced apoptosis is related to activation of MEK/ERK pathway to promote Bax transfer from cytoplasm to mitochondria, which is consistent with our results. We hypothesized that sevoflurane inhibits Bcl-2 by inhibiting the activation of the ERK pathway to activate the mitochondrial apoptotic pathway to induce apoptosis.

The mechanism of how the Ras/Raf/MEK/ERK pathway regulates autophagy has received extensive attention recently. There are a variety of anti-tumor drugs that target the inhibition of the ERK signaling pathway, such as sorafenib, vemurafenib and dabrafenib. With the deepening of research, the role of inhibiting ERK signaling pathway in the positive regulation of autophagy has also been gradually discovered. For example, gefitinib can down-regulate the expression of ERK pathway proteins and induce autophagy in U87 cells [45] and NSCLC cells [46]. Ras inhibitor salirasib can promote autophagy in Hela cells, HCT-116 cells and DLD-1 cells [47]. Raf kinase inhibitor sorafenib can cause autophagy in DU145 cells and HepG2 cells [48]. We also found that sevoflurane upregulates the expression of autophagy marker proteins LC3 and Beclin 1 to promote autophagy in SW480 and SW620 cells. Studies have shown that the ERK pathway can activate ERK directly under stress conditions to directly promote the up-regulation of LC3 and p62 to initiate autophagy [49]. On the other hand, ERK pathway can down-regulate the expression of lysosomal-associated membrane protein Lamp 1 (LAMP1) and LAMP2 to prevent the binding of autophagosomes to lysosomes to inhibit the degradation of autophagosomes [50]. In addition, some scholars have confirmed that activated ERK signaling pathway can inhibit autophagy indirectly by activating the PI3K/Akt/mTOR pathway [51]. However, the mechanisms involved in the regulation of autophagy by the ERK pathway are extremely complex and sometimes seem contradictory. Therefore, how sevoflurane fine-tunes the mechanism of autophagy needs to be further explored.

ERK signaling pathway activation also can increase cell invasiveness by regulating EMT topromote cancer cell invasion [52]. Additionally , inhibition of phosphorylation of ERK can down-regulate the expression of MMPs at the transcriptional level and inhibit the invasion and metastasis of tumor cells [53]. We also found that sevoflurane inhibited MMPs expression and EMT by inhibiting ERK activation to inhibit cell invasion. However, the mechanism of action of ERK pathway on EMT regulation is unclear. Twist, a novel zinc finger transcription factor, was suggested to be an important inducer of EMT [54]. Li et al. [55] demonstrated that activation of ERK pathway can result in Twist activation and promote Bmi expression to futher EMT initiation and cellular migration. MAPK/ERK pathway can lead to the activation of the transcription factor Snail, a known regulator of EMT in a number of cellular, to regulate EMT [56]. In the follow-up study, we also need to explore the role of upstream regulators of EMT, such as Snail and Twist, to explore the mechanism of sevoflurane regulation of EMT. Taken together, we believe that sevoflurane can participate in the regulation of proliferation, apoptosis and invasion of colon cancer cells by inhibiting the activation of EKR signaling pathway (Fig. 5).

Kvolik S et al. [22] found that sevoflurane has anti-ancer effects on human colon cancer (Caco-2), human laryngeal carcinoma (HEp-2), human pancreatic cancer cells (MIA PaCa-2), colon cancer lymph node metastasis of poorly differentiated cells (SW-620). Additionally, the anticancer effect of sevoflurane was observed in liver cancer [57], lung cancer [58], breast cancer [59], and gastric cancer [60]. Watanabe et al. [61] and Suter et al. [62] found that sevoflurane did not affect the biological characteristics of normal small airway epithelial cells (SAEC) and normal alveolar epithelial cells, but when normal cells are stimulated by tumor necrosis factor (TNF)-α or lipopolysaccharide (LPS), sevoflurane begins to exert its effects. Recently, Shi et al. [63] reported that investigated the effect of sevoflurane on glioma stem cells(GSCs) in vitro and found that sevoflurane can enhance tumor growth through tumor stem cells. Lucchinetti et al. [64] found that sevoflurane preconditioning promotes growth and proliferation of stem cell-like human endothelial progenitors. Simultaneously, the improvement of nerve damage by sevoflurane was also confirmed in the study of nerve cells [65,66]. Therefore, we speculate that sevoflurane may not be specific to specific cancer cell subsets. However, the effects of sevoflurane on different cells need to be further explored.

5. Conclusion

The present study demonstrated that Sevoflurane can inhibit the proliferation and invasion of colon cancer cellswhile promotes apoptosis in vitro. In addition, the effect of sevoflurane on cell invasion may be related to its regulation of EMT. SW480 cell xenograft tumor experiments also showed that sevoflurane inhibited the tumorigenic ability of cancer cells *in vivo* (Fig. 6). One possible mechanism of the anti-tumor effect of sevoflurane in colon cancer cells may be related to its regulation of ERK signaling pathway. This provides a new basis for the clinical application of sevoflurane in the treatment of colon cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YX, ZYT and RW conceptualized and designed the study. YX performed the cell culture and most of the experiments and statistical analysis as well as drafted the manuscript. ZYT helped with transwell assay and Western blot. RW made significant contributions to editing and revising the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Declaration of competing interest

The authors report no conflicts of interest in this work.

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