Pre-Clinical Research Report

Journal of INTERNATIONAL MEDICAL RESEARCH

Journal of International Medical Research
49(4) 1–12

DOI: 10.1177/03000605211005936
journals.sagepub.com/home/imr
SAGE

Sevoflurane represses the migration and invasion of gastric cancer cells by regulating forkhead box protein 3

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Abstract

Objective: Previous studies suggested that sevoflurane exerts anti-proliferative, anti-migratory, and anti-invasive effects on cancer cells. To determine the role of sevoflurane on gastric cancer (GC) progression, we evaluated its effects on the proliferation, migration, and invasion of SGC7901, AGS, and MGC803 GC cells.

Methods: GC cells were exposed to different concentrations of sevoflurane (1.7, 3.4, or 5.1% v/v). Cell viability, migration, and invasion were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and Transwell assays. Immunohistochemical staining and immunoblotting were performed to analyze forkhead box protein 3 (FOXP3) protein expression in tissue specimens and cell lines, respectively.

Results: FOXP3 was downregulated in human GC specimens and cell lines. Functionally, FOXP3 overexpression significantly inhibited the proliferation, migration, and invasion of GC cells and accelerated their apoptosis. Moreover, sevoflurane significantly blocked GC cell migration and invasion compared with the findings in the control group. However, FOXP3 silencing neutralized sevoflurane-induced apoptosis and the inhibition of GC cell migration and invasion. Sevoflurane-induced apoptosis and the suppression of migration and invasion might be associated with FOXP3 overactivation in GC cells.

Conclusions: Sevoflurane activated FOXP3 and prevented GC progression via inhibiting cell migration and invasion *in vitro*.

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Keywords
Gastric cancer, sevoflurane, FOXP3, prognosis, migration, invasion, proliferation, apoptosis

Date received: 22 February 2021; accepted: 5 March 2021

Introduction

Volatile anesthetics (VAs), such as desflurane and sevoflurane, play multiple roles in tumor progression.[1] Compelling evidence suggests that VAs activate or suppress the immune system, revealing their beneficial and detrimental effects on carcinogenesis.[1,2] For example, sevoflurane preconditioning decreases the invasiveness of colorectal cancer cells by repressing matrix metalloproteinase-9.[3] Meanwhile, sevoflurane stimulation enhanced proliferation in six of eight cancer cell lines (NCI-H1299, MDA-MB-231, HCT116, DLD-1, HT29, and RKO); however, the drug repressed cell proliferation in A549 and MCF-7 cells.[4] At present, additional research is needed to determine the roles and underlying molecular mechanisms of sevoflurane in gastric cancer cell growth.

It is increasingly recognized that sevoflurane mediates multiple signaling pathways, including the mitochondrial apoptosis pathway, mitogen-activated protein kinase pathways, and transcription factor pathways to adjust the balance between cell proliferation and apoptosis.[5,6] Forkhead box protein 3 (FOXP3) was originally identified as a transcription factor important for the function of lymphocytes, especially $CD4^+CD25^+$ regulatory T cells.[7,8] Moreover, FOXP3 is widely expressed in human epithelial cells, and it promotes epithelial proliferation to repair tissue.[9,10] In human tissues of aggressive cancers, including non-small cell lung cancer, cervical cancer and head and neck cancer,[11–13] FOXP3 upregulation is associated with tumorigenesis, metastasis, and poor prognosis. Conversely, FOXP3 is downregulated in ovarian cancer, hepatocellular carcinoma (HCC), and prostate cancer.[14–17] Contradictorily, overexpression of FOXP3 augments or hampers cell proliferation and apoptosis in various cancer cells.[11,14,16] It was recently found that the functions of FOXP3 are also controversial in the progression of gastric cancer (GC).[18–20] For instance, overexpression of FOXP3 suppresses GC cell growth *in vivo*,[18] and silencing FOXP3 expedites the proliferation of GC cells *in vitro*.[19] By contrast, FOXP3 is upregulated in GC tissues, and overexpression of FOXP3 facilitates cell proliferation, migration, and invasion. These effects are reversed by FOXP3 loss-of-function mutations in GC cells.[20] It is reasonable to believe that FOXP3 is closely related with gastric carcinogenesis.

In the present study, we examined the effects of sevoflurane on GC cell proliferation, migration, and invasion. In addition, the association of sevoflurane and FOXP3 and its clinical significance in the progression of GC were delineated. A better understanding of sevoflurane-modulated FOXP3 might offer a novel avenue for the treatment of GC.

Materials and methods

Clinical samples and cell culture

Human GC tissues and adjacent non-tumor tissues were collected from 61 patients with GC undergoing surgery at The Fourth

Hospital of Hebei Medical University. The clinical research was approved by the Ethics Committee of The Fourth Hospital of Hebei Medical University. Informed consent was obtained from all patients prior to sample collection. The normal human gastric cell line HEGC and GC cell lines (SGC7901, MKN28, AGS, MKN45, and MGC803) were purchased from Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences (Shanghai, China) and cultured with RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, USA). GC cells were exposed to sevoflurane (Merck KGaA, Darmstadt, Germany) at low (1.7% v/v), medium (3.4% v/v), or high concentrations (5.1% v/v), as described previously.[21,22]

Immunohistochemical (IHC) staining and immunoblotting

The experimental procedures of IHC staining and immunoblotting were described previously.[23] The primary antibody against FOXP3 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA, cat. no: sc-166212; dilution: 1:100 for IHC staining 1:1000 for immunoblotting). FOXP3-positive staining was evaluated using Image Pro-Plus 6 software (Media Cybernetics, Inc., Rockville, MD, USA). The blots were developed using an ECL chemiluminescence kit (Santa Cruz Biotechnology). Quantitative data were analyzed using Quantity One® software version 4.5 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

RT-qPCR. RNA extraction was performed using TRIzol (Invitrogen; Thermo Fisher Scientific). Synthesis of cDNA was performed via reverse transcription using 2 μg of total RNA, Moloney murine leukemia virus reverse transcriptase (Invitrogen; Thermo Fisher Scientific), and oligo dT (15) primers (Fermentas; Thermo Fisher Scientific) according to the manufacturer's protocol. PCR was performed using a DNA engine (PCR system 9700, Applied Biosystems, Thermo Fisher Scientific) and TaqMan Universal PCR Master Mix (Thermo Fisher Scientific). GAPDH was used as an internal control to normalize the data to determine the relative expression of FOXP3. The PCR primers used in this study were as follows: FOXP3 forward, 5′-GTGGCCCGGATGTGAGAAG-3′; FOXP3 reverse, 5′-GGAGCCCTTGTCGGATGATG-3′; GAPDH forward, 5′-AGCCACATCGCTCAGACAC-3′; and GAPDH reverse, 5′-GAAGGTGAAGGTCGGAGTC-3′.

Cell transfection

Short hairpin RNA (shRNA) was designed to silence the expression of FOXP3 and synthesized by GenePharm (Shanghai, China). Sh-FOXP3 was transfected into SGC7901, AGS, and MGC803 cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific) according to the manufacturer's protocols. FOXP3 expression plasmids (vector-FOXP3) and a negative-control empty plasmid (vector-con) were purchased from GeneCopoeia, Inc. (Rockville, MD, USA) and then transfected into SGC7901, AGS, and MGC803 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols.

Analysis of cell viability using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell (2×10^5/well) viability was measured using an MTT Cell Proliferation/Viability Assay kit (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol.

Transwell assays

Using SGC7901, AGS, and MGC803 cells, Transwell plates (8-μm pore size) without

Matrigel were used for the Transwell migration assay, and Transwell plates (8-μm pore size) with Matrigel were used for the Transwell invasion assay, as described previously.[24]

TUNEL assay

The TUNEL assay was performed using a TUNEL kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China; cat. no: G002-1-2) as described previously.[25] Cell apoptosis was analyzed as the proportion of TUNEL-positive staining cells.

Statistical analysis

Data were presented as the mean ± standard deviation. Statistical analysis was performed using IBM SPSS Statistics Version 19.0 (IBM, Armonk, NY, USA). Student's *t*-test was used to analyze between-group differences. Inter-group differences were analyzed via one-way analysis of variance. Overall survival was analyzed using the Kaplan–Meier method. $P < 0.05$ indicated statistical significance.

Results

FOXP3 expression was diminished in GC tissues and cell lines

As presented in Figure 1a, FOXP3 was widely distributed in the cytoplasm and on the basal side of the mucosal layer in paracancerous tissues; however, the positivity rate for FOXP3 was markedly lower in GC tissues. The tissue microarray of IHC staining revealed that FOXP3 expression was significantly lower in GC tissues than in the corresponding normal paracancerous tissues (Figure 1b), reflecting that the expression of FOXP3 was downregulated in 43 of 61 GC tissues (70.5%, Figure 1c). RT-qPCR and western blotting were used to detect the mRNA and protein expression, respectively, of FOXP3 in normal human gastric cells and GC cell lines. FOXP3 mRNA and protein expression was significantly downregulated in three of five GC lines (SGC7901, AGS, and MGC803) compared with that in normal gastric cells, but no obvious change was noted in MKN28 and MKN45 cells (all $P < 0.01$, Figure 1d and 1e). Therefore, we further examined the function of FOXP3 in SGC7901, AGS, and MGC803 cells.

Low FOXP3 expression was associated with poor prognosis in patients with GC

Based on the proportion of FOXP3-positive staining in GC tissues, patients with GC were divided into high (n = 40) and low expression groups (n = 21). Kaplan–Meier analysis revealed worse overall survival ($P < 0.05$, Figure 2a) and disease-free survival ($P < 0.05$, Figure 2b) in patients with low FOXP3 expression.

FOXP3 overexpression repressed the proliferation, migration, and invasion of GC cells in vitro

To investigate the roles of FOXP3 in GC cell proliferation, migration, and invasion, FOXP3 plasmids were stably transfected into SGC7901, AGS, and MGC803 cells. As illustrated in Figure 3a, FOXP3-transfected GC cells exhibited significantly higher FOXP3 protein expression than vector-transfected cells (all $P < 0.05$). Starting 48 hours after FOXP3 transfection, cell viability was evaluated by the MTT assay after an additional 24, 48, or 72 hours of incubation. The MTT assay revealed that GC cell proliferation was inhibited by FOXP3 overexpression *in vitro* (all $P < 0.001$, Figure 3b). As presented in Figure 3c and 3d, FOXP3 overexpression significantly slowed the migration and invasion of SGC7901,

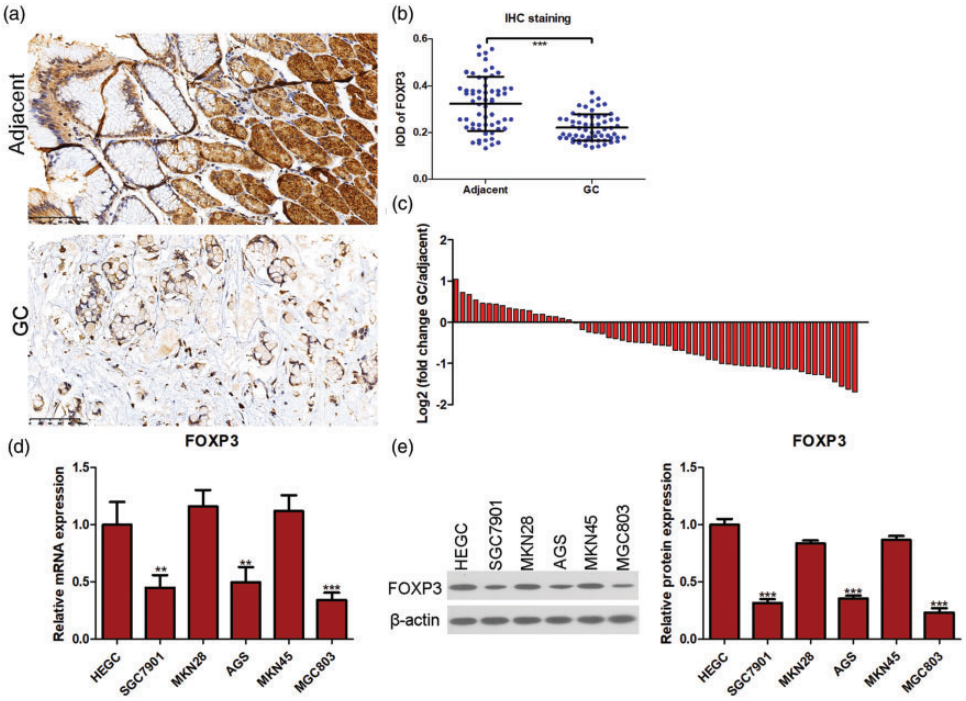

Figure 1. FOXP3 expression was decreased in GC tissues and cell lines. IHC staining and Image Pro-Plus 6 software were used to evaluate FOXP3 expression in GC tissues and adjacent non-tumor tissues (a and b). FOXP3 was downregulated in 43 of 61 (70.5%) GC tissues compared with its expression in adjacent non-tumor tissues (c). RT-qPCR and western blotting were performed in GC cell lines to estimate FOXP3 mRNA and protein expression, respectively (d and e). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the corresponding control group.
FOXP3, forkhead box P3; GC, gastric cancer; IHC, immunohistochemical.

AGS, and MGC803 cells compared with the findings for vector-transfected cells. The results of the TUNEL assay indicated that FOXP3 overexpression led to a significant increase in the number of TUNEL-positive SGC7901, AGS, and MGC803 cells (all $P < 0.05$, Figure 4a and 4b).

Sevoflurane repressed the migration and invasion of GC cells in vitro *by activating FOXP3*

Concerning the effects of sevoflurane on FOXP3 expression, western blotting indicated that its protein expression was significantly increased by 12 hours of sevoflurane stimulation in SGC7901, AGS, and MGC803 cells in a concentration-dependent manner (all $P < 0.05$, Figure 5a). Conversely, MTT assays suggested that sevoflurane exposure for 12 hours had no obvious effect on cell viability *in vitro* (Figure 5b). Intriguingly, sevoflurane treatment for 12 hours significantly blocked GC cell migration and invasion compared with the control findings (all $P < 0.05$, Figure 5c and 5d). However, FOXP3 silencing neutralized the sevoflurane-induced inhibition of GC cell migration and invasion (all $P < 0.05$, Figure 5c and 5d). Furthermore, sevoflurane treatment significantly increased the proportion of TUNEL-positive SGC7901, AGS, and MGC803 cells, whereas FOXP3 silencing partially

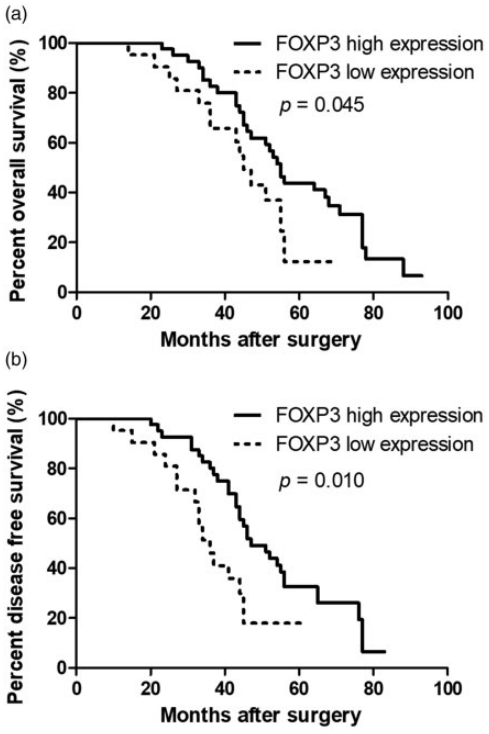

Figure 2. Low FOXP3 expression was associated with poor prognosis in patients with GC. Based on the level of FOXP3-positive staining in GC tissues, patients with GC were divided into high (n = 40) and low expression groups (n = 21). The Kaplan–Meier method was used to investigate the associations of FOXP3 expression with overall survival (a) or disease-free survival (b) in patients with GC. FOXP3, forkhead box P3; GC, gastric cancer.

counteracted the pro-apoptotic effects of sevoflurane on GC cell lines *in vitro* (all $P < 0.05$, Figure 6a and 6b).

Discussion

Clinically, anesthetic usage potentially affects outcomes in the perioperative period in patients with cancer.[1] Anesthetics can directly or indirectly regulate tumor cell migration or invasion, thereby expediting the malignant process in many cancers.[26] For example, in, SW620 and HCT116 colorectal cancer cells, exposure to 4% sevoflurane led to the significant inhibition of migration and invasion.[27] Liang *et al.*[26] revealed an anti-invasion effect of sevoflurane on A549 cells. In addition, anesthetics modulate immune cells, including neutrophils, macrophages, natural killer cells, T cells, and B cells, and phenotypes responsible for the immunosuppressive or immunoactivating effects indirectly activate or hinder the migration and invasion of cancer cells.[1] At present, the effects of sevoflurane on GC cell proliferation, migration, and invasion have not been completely illuminated. Therefore, our study explored whether sevoflurane exhibited anti-neoplastic activity in GC cells.

First, the proliferative activity of GC cells, including SGC7901, AGS, and MGC803 cells, was not changed by 5.1% sevoflurane stimulation for 12 hours. These findings were consistent with previous reports that stimulation with 4% sevoflurane for 8 hours did not alter the viability of SW620 and HCT116 cells.[27] In addition, we also found that the inhibition efficiency of 5.1% sevoflurane on GC cell migration and invasion exceeded 50%. Previous studies reported that sevoflurane suppresses migration and invasion in multiple cancers, including lung cancer, colorectal cancer, and glioma.[26–28] We revealed for the first time in this study that sevoflurane has the potential to inhibit migration and invasion in GC.

Mechanically, we hypothesized that FOXP3 might be a potential target through which sevoflurane inhibits migration and invasion of GC. FOXP3 protein expression was measured using western blotting in GC cells after exposure to various concentrations of sevoflurane. *In vitro* experiments revealed a significant increase of FOXP3 protein expression in GC cells after treatment with sevoflurane. Subsequently, GC cells were concomitantly subjected to sevoflurane stimulation and sh-FOXP3

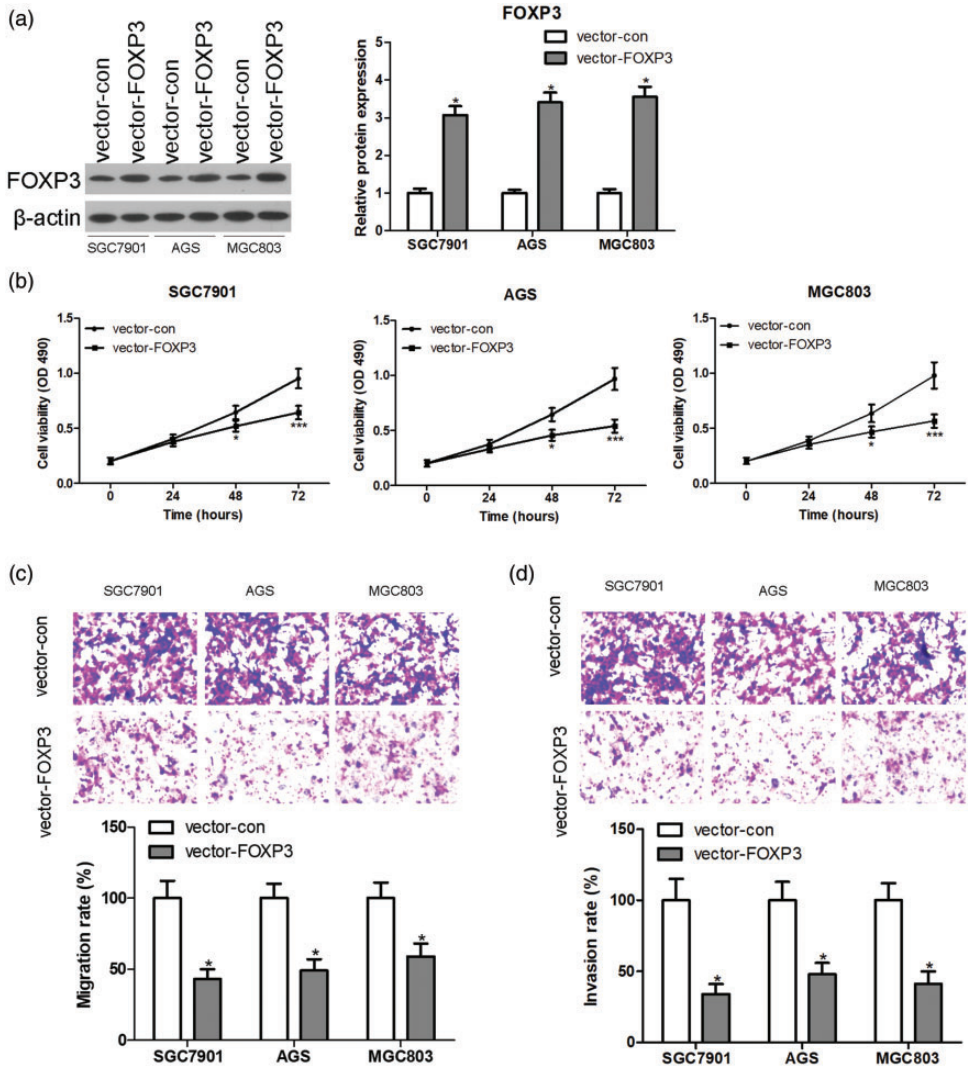

Figure 3. FOXP3 overexpression repressed the proliferation, migration, and invasion of GC cells *in vitro*. After the transfection with vector-con or vector-FOXP3 overexpression plasmids into SGC7901, AGS, and MGC803 cells, the protein expression of FOXP3 was measured using western blotting (a). Cell viability (b), migration (c), and invasion (d) were evaluated using the MTT and Transwell assays, respectively. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with the corresponding control group.

FOXP3, forkhead box P3; GC, gastric cancer; vector-con, negative-control empty plasmid; vector-FOXP3, FOXP3 overexpression plasmid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

transfection, and the sevoflurane-induced suppression of GC cell migration and invasion was counteracted by FOXP3 silencing. Our present study indicated that the potential mechanisms of the sevoflurane-induced suppression of migration and invasion might be associated with FOXP3 overactivation in GC cells.

FOXP3 functions as a tumor suppressor or oncogene in varying cancers based on

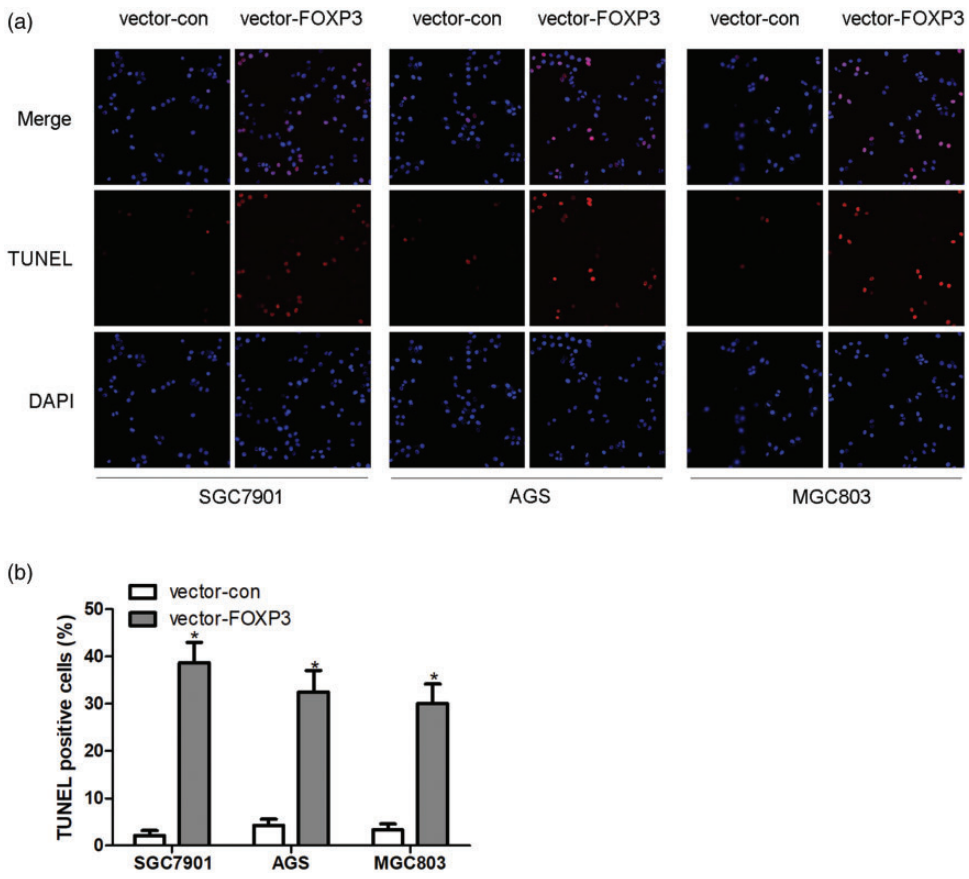

Figure 4. FOXP3 overexpression induced apoptosis in GC cells *in vitro*. After transfection with vector-con or vector-FOXP3 into SGC7901, AGS, and MGC803 cells, the TUNEL assay was performed to evaluate cell apoptosis (a and b). $^{*}P < 0.05$ compared with the corresponding control group.
FOXP3, forkhead box P3; GC, gastric cancer; vector-con, negative-control empty plasmid; DAPI, 4′,6-diamidino-2-phenylindole.

in vitro models.[29] Zuo et al.[30] validated that FOXP3 overexpression inhibited colony formation by human breast cancer cells *in vitro*. FOXP3 upregulation represses proliferation, migration, and invasion in epithelial ovarian cancer by reducing the expression of matrix metalloproteinase-2, a key factor promoting the invasion and metastasis of cancer.[14] Shi *et al.*[15] corroborated that FOXP3 could suppress the proliferation and invasion of HCC cells *in vitro*. Based on the aforementioned data, FOXP3 may function as a tumor suppressor *in vitro*. Oppositely, FOXP3 overexpression promotes cell proliferation, cell invasion, and epithelial–mesenchymal induction in non-small cell lung cancer.[11] FOXP3 is upregulated in thyroid cancer cells, and FOXP3 silencing leads to the inhibition of proliferation and migration and accelerates apoptosis, indicating that FOXP3 plays a positive role in thyroid cancer growth.[31] These findings suggest that the function of FOXP3 in cancer cells remains controversial given its ability to function as both a tumor suppressor and oncogene.

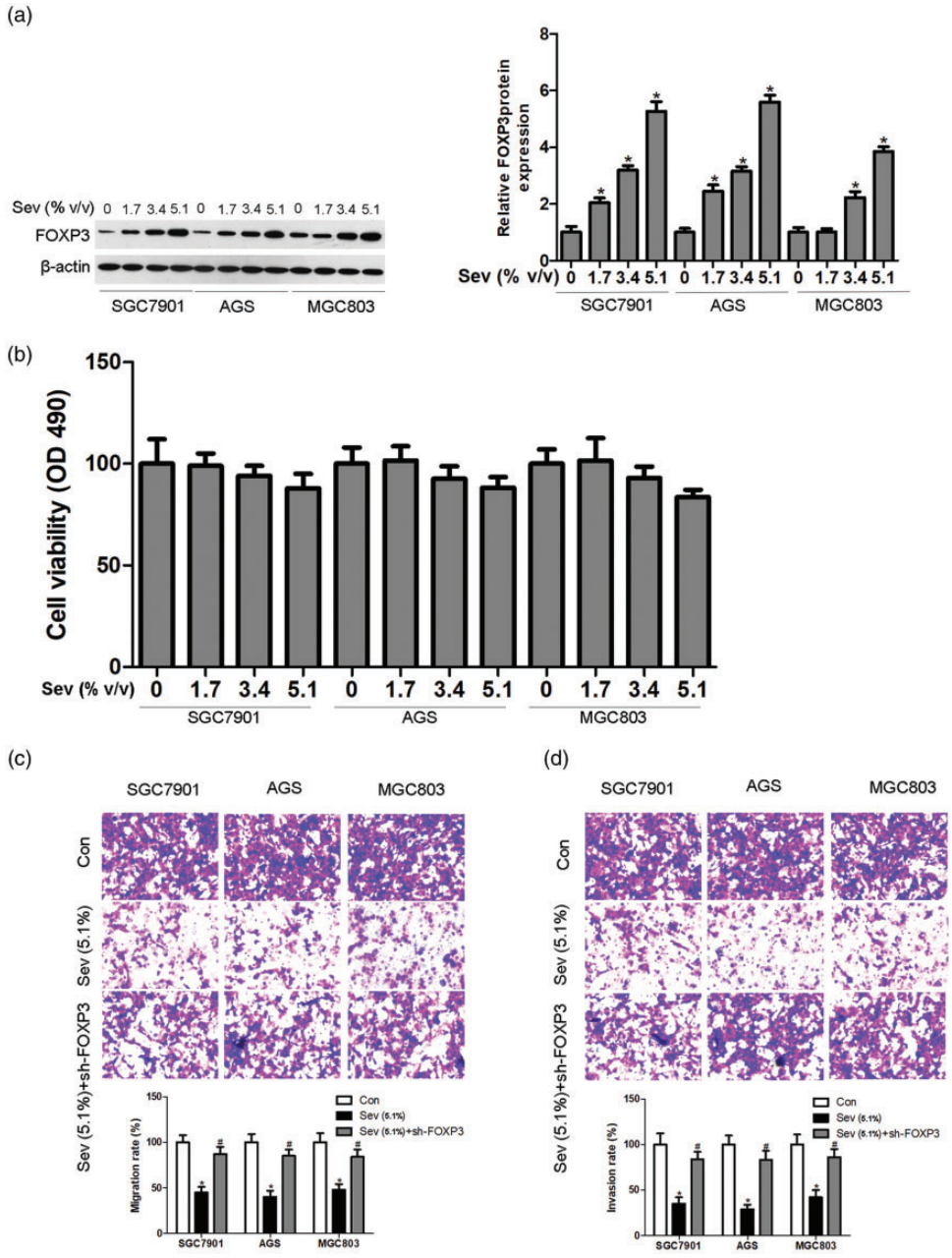

Figure 5. Sevoflurane repressed the migration and invasion of GC cells *in vitro* by activating FOXP3. The protein expression of FOXP3 in response to sevoflurane stimulation was measured using western blotting (a). The viability of SGC7901, AGS, and MGC803 cells in response to sevoflurane stimulation was analyzed using the MTT assay (b). After GC cells were treated with sevoflurane and subjected to sh-FOXP3 transfection for 12 h, cell migration (c) and invasion (d) were evaluated using Transwell assays. $^{*}P < 0.05$ compared with the corresponding control group; $^{\#}P < 0.05$ compared with the sevoflurane-treated group.
GC, gastric cancer; FOXP3, forkhead box P3; sh-FOXP3, short hairpin RNA targeting FOXP3; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sev, sevoflurane; OD, optical density.

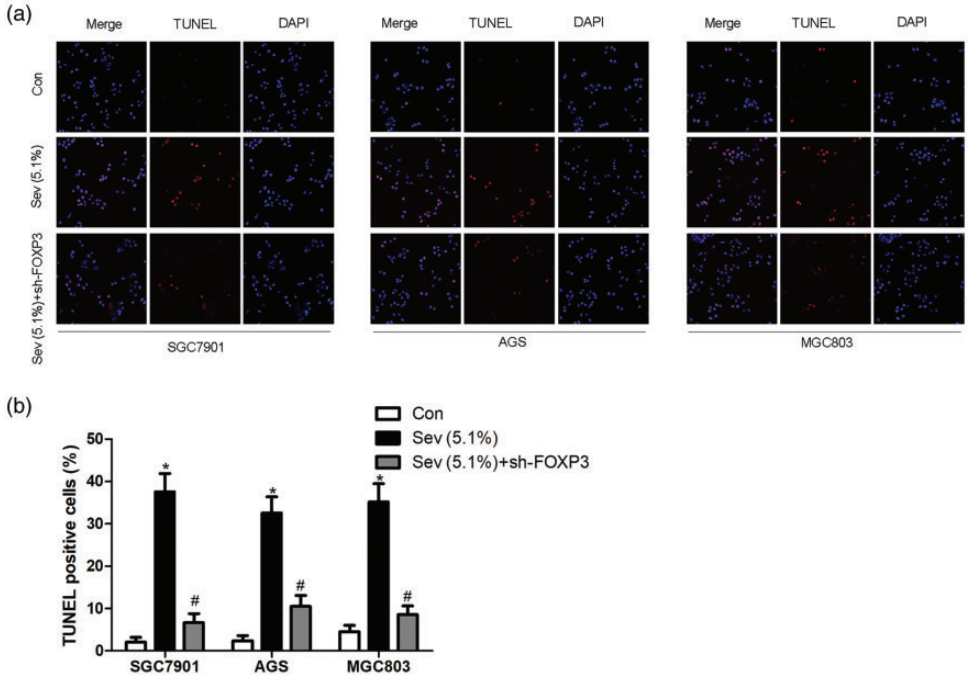

Figure 6. Sevoflurane-induced apoptosis was reversed by sh-FOXP3 transfection in GC cells. After GC cells were treated with sevoflurane and subjected to sh-FOXP3 transfection for 12 h, the TUNEL assay was performed to evaluate cell apoptosis (a and b). $^{*}P < 0.05$ compared with the corresponding control group; $^{\#}P < 0.05$ compared with the sevoflurane-treated group.
GC, gastric cancer; sh-FOXP3, short hairpin RNA targeting forkhead box P3; DAPI, 4′,6-diamidino-2-phenylindole.

In GC, FOXP3 gain-of-function mutations block proliferation and facilitate apoptosis in AGS and MKN45 cells.[32] Li *et al.*[33] also found that FOXP3 silencing accelerates the proliferation of MGC80-3 cells. In our study, the downregulation of FOXP3 was detected in human GC specimens and cell lines. Functionally, FOXP3 overexpression significantly inhibited proliferation and impeded migration and invasion in SGC7901, AGS, and MGC803 GC cells.

In conclusion, the sevoflurane-induced inhibition of GC cell migration and invasion might be associated with FOXP3 upregulation. Sevoflurane as an activator of FOXP3 is proposed as a novel treatment to prevent GC progression *in vitro* by inhibiting cell migration and invasion.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Authors' contributions

Study design: FY and HJ; literature research: FY, HW, CL, and HJ; data acquisition and analysis: FY, HW, CL, and HJ; manuscript

preparation: FY, HW, and CL; manuscript editing: HJ; manuscript review: HJ; cell experiments: FY, HW, CL, and HJ; final approval of the version to be published: FY, HW, CL, and HJ.

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