ANESTHESIOLOGY

Sevoflurane Enhances Proliferation, Metastatic Potential of Cervical Cancer Cells *via* the Histone Deacetylase 6 Modulation *In Vitro*

Wenwen Zhang, M.D., Ph.D., Bo Sheng, M.D., Sisi Chen, M.B., Hailin Zhao, B.Sc., Ph.D., Lingzhi Wu, B.Sc., M.Res., Yibing Sun, M.D., Ph.D., Jiang Cui, B.Sc., M.Res., Xueqiong Zhu, M.D., Ph.D., Daqing Ma, M.D., Ph.D., F.R.C.A.

ANESTHESIOLOGY 2020; 132:1469–81

EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Preclinical investigations have shown a variety effects of anesthetics on cancer cell biology
- The effects of the volatile anesthetic sevoflurane on cervical cancer cells has not been previously reported

What This Article Tells Us That Is New

- Sevoflurane enhances the malignant potential of two immortalized cervical cancer cell lines *in vitro*
- The underlying mechanisms include sevoflurane-induced increase in histone deacetylase 6, which, *via* changes in cellular cytoskeleton dynamics, may promote the invasive properties of cervical cancer cells

ABSTRACT

Background: Sevoflurane is commonly used for cervical cancer surgery, but its effect on cervical cancer cell biology remains unclear. This mechanistic study explores how sevoflurane affects the proliferation and metastatic potential of immortalized cervical cancer cell lines.

Methods: Cultured cervical cancer Caski and HeLa lines were exposed to 1, 2, or 3% sevoflurane for 2 or 4 h. Cell proliferation was determined through the Kit-8 assay and Ki-67 immunofluorescent staining. Cell migration and invasion were evaluated with the Transwell assay. Immunofluorescent staining and Western blot analysis were used to identify sevoflurane-induced morphological and biochemical changes.

Results: Sevoflurane exposure for either 2 or 4 h significantly increased HeLa cell proliferation in a time- and concentration-dependent manner to be 106 ± 2.7% and 107 ± 1.4% relative to the controls (n = 10; $P = 0.036$; $P = 0.022$) at 24 h after exposure and to be 106 ± 2.2% and 106 ± 1.7% relative to the controls (n = 10; $P = 0.031$; $P = 0.023$) at the highest concentration of 3% sevoflurane studied, respectively, but not Caski cells. Sevoflurane promoted invasion ability (1.63 ± 0.14 and 1.92 ± 0.12 relative to the controls) and increased cell size (1.69 ± 0.21 and 1.76 ± 0.13 relative to the controls) of Caski and HeLa cells (n = 6; all $P < 0.001$), respectively. Sevoflurane increased histone deacetylase 6 expression in both cells, and histone deacetylase 6 knockdown abolished the prometastatic effects of sevoflurane. Sevoflurane also induced deacetylation of α-tubulin in a histone deacetylase 6–dependent manner. The protein kinase B (AKT) or extracellular regulated protein kinase (ERK1/2) phosphorylation inhibition attenuated sevoflurane-induced histone deacetylase 6 expression.

Conclusions: Sevoflurane enhanced proliferation, migration, and invasion of immortalized cervical cancer cells, which was likely associated with increasing histone deacetylase 6 expression caused by phosphatidylinositide 3-kinase/AKT- and ERK1/2-signaling pathway activation.

(ANESTHESIOLOGY 2020; 132:1469–81)

Cervical cancer is the second largest class of gynecologic malignancy globally, with 88% of deaths estimated to occur in developing countries.[1] The traditional therapy of cervical cancer is surgery or radiotherapy.[2] The leading cause of death is metastatic recurrence after surgery in cervical cancer patients.[3] It has been reported that perioperative factors including anesthetics and anesthesia technique may affect cancer cell biology and hence increase recurrence risk.[4] Inhalational anesthetic desflurane was documented to be related to improved disease-free survival of the patients with stage III ovarian cancer undergoing primary cytoreductive surgery.[5] However, the direct influence of anesthetics, especially inhalational anesthetics, on cancer cell biology is not only limited but also contradictory.[6–9] Indeed, it was demonstrated that volatile anesthetic sevoflurane enhanced proliferation and migration of breast cancer.[6] In contrast, it was also revealed that sevoflurane inhibited p38 mitogen–activated protein kinase pathway, which is involved in the down-regulation of matrix metalloproteinase 2, matrix metalloproteinase 9, fascin, and ezrin, thus suppressing the

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org). X.Z. and D.M. contributed equally to this work.

Submitted for publication November 29, 2018. Accepted for publication December 11, 2019. Published online first on January 28, 2020. From the Department of Obstetrics and Gynecology, the Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, China (W.Z., B.S., S.C., X.Z.); Anesthetics, Pain Medicine and Intensive Care, Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, Chelsea and Westminster Hospital, London, United Kingdom (W.Z., H.Z., L.W., Y.S., J.C., D.M.); and the Department of Anesthesiology and Critical Care Medicine, Peking University First Hospital, Beijing, China (Y.S.)

 Anesthesiology 2020; 132:1469–81. DOI: 10.1097/ALN.0000000000003129

<zdoi;. DOI: 10.1097/ALN.0000000000003129>

migration and invasion of lung cancer cells.[8] In addition, it has been also shown that sevoflurane can promote the metastatic potential of renal carcinoma but not of non–small cell lung cancer in *in vitro*.[9] In this regard, the effect of volatile anesthetics on cervical cancer cell proliferation and metastatic potential remains unknown.

Histone deacetylase 6 is a member of class II histone deacetylases family involved in multiple cellular processes related to cancer including tumorigenesis, carcinogenic transformation, motility, stress response, anchorage-independent proliferation, tumor aggressiveness, and a myriad of other signaling pathways.[10–12] The histone deacetylase 6 level is likely regulated by the phosphatidylinositide 3-kinase/protein kinase B (AKT) or extracellular regulated protein kinase (ERK1/2) pathways, because AKT increases histone deacetylase 6 activity in vascular smooth muscle cells,[13] and histone deacetylase 6 function was activated by ERK1/2, thus enhancing cell migration.[14] Histone deacetylase 6 deacetylates α-tubulin that dimerizes with β-tubulin to form the crucial dynamic parts of cytoskeleton microtubules.[15] The expression of histone deacetylase 6 has been shown to increase in many cancers including cervical cancer.[16,17] It was reported that knockdown of histone deacetylase 6 suppressed proliferation of HeLa cells and induced apoptosis.[16] It has been documented that inhalational anesthetic isoflurane increased the expression of histone deacetylase 2 and histone deacetylase 3, leading to neuronal apoptosis of neonatal rats.[18] It could be postulated that overexpression of histone deacetylase 6 by anesthetics in patients with cancer may lead to adverse outcomes.

This study was designed to investigate the potential impact of sevoflurane on the proliferation, migration, and invasion of immortalized cervical cancer cell lines including squamous cancer cells Caski and adenocarcinoma cells HeLa *in vitro* and the underlying mechanisms with focus on the histone deacetylase 6 pathway as we hypothesized that sevoflurane may activate the histone deacetylase 6 pathway, therefore promoting metastatic potential of cervical cancer cells.

Materials and Methods

Cell Lines and Cell Culture

Human immortalized cervical cancer Caski (catalog number 87020501; squamous cancer; passaged 10 times) and HeLa (catalog number 93021013; pure epitheloid carcinoma cells not derivatives; passaged fewer than 10 times) cells were purchased from European Collection of Authenticated Cell Cultures. Both cells were authenticated by assessing short tandem repeat DNA profiles, thawed, used within 6 months of receipt, and passaged less than 20 times. Caski cells was maintained in RPMI 1640 (Gibco, Invitrogen, USA) supplemented with 10% fetal bovine serum (Gibco, Invitrogen). HeLa was cultured in Dulbecco's modified Eagle's medium (Gibco, Invitrogen) containing 10% fetal bovine serum. The cells were maintained in a humidified incubator supplying with 5% CO_2 in air at 37°C. Once ready, they were randomly used for experiments in an unblinded manner.

Sevoflurane Exposure

HeLa or Caski cells were placed in a purposely built airtight gas chamber with inlet and outlet connectors. Sevoflurane, mixed with 5% CO_2 balanced in air, was delivered into the chamber by a vaporizer (sevoflurane; Abbott, USA) at a rate of 2 l/min for a maximum of 5 min until the desired gas concentration was reached. The concentrations of sevoflurane in the chamber were monitored using an anesthetic monitor (Datex-Ohmeda, United Kingdom) at the outlet part of chamber. The chamber was then moved into a 37°C incubator (Galaxy R CO_2 chamber; New Brunswick Scientific, USA) for 2 or 4 h, which was the common duration of cervical cancer surgery. To simulate the anesthesia condition of cervical cancer surgery, cervical cancer cells were treated with three commonly used concentrations of sevoflurane (1, 2, or 3%), about equivalent to 0.5, 1, and 2 minimum alveolar concentrations, respectively. The control group was treated with 5% CO_2 balanced with air, and the sevoflurane groups were exposed to 1, 2, or 3% sevoflurane mixed with 5% CO_2 balanced with air.

Immunofluorescent Staining

The cells were cultured at a density of 1.5×10^4 cells/well in 24-well plate. Then cells were fixed in 4% paraformaldehyde for 15 min, washed with phosphate-based saline, permeabilized with 0.1% Triton X-100 in phosphate-based saline for 30 min, and blocked in 10% goat serum in phosphate-based saline for 1 h at room temperature. Subsequently, the cells were incubated overnight at 4°C with rabbit anti-Ki-67, rabbit anti–histone deacetylase 6, rabbit anti–matrix metalloproteinase 9, rabbit anti–E-cadherin, rabbit anti–α-tubulin, mouse anti–F-actin, or mouse anti–acetylated α-tubulin (1:200 dilution; Cell Signaling Technology, USA). After washing with phosphate-based saline, the cells were incubated with fluorescein isothiocyanate–conjugated or rhodamine-conjugated secondary antibody (1:400) for 1 h and finally stained with 4,6-diamidino-2-phenylindole for nuclei staining. The cells were visualized, and images were captured with a fluorescence microscope (Olympus, Japan). For cytoskeletal rearrangement, images of cells triple-labeled with rhodamine–F-actin, fluorescein isothiocyanate–α-tubulin, and 4,6-diamidino-2-phenylindole were captured, and six triple-labeled cells were chosen to analyze cell area by using ImageJ 1.35 software (National Institutes of Health, USA).

Cell Viability Assay

The cell counting kit 8 (Sigma, United Kingdom) was used to analyze the viability of cervical cancer cells after sevoflurane treatment. The cells were seeded into 96-well plates

(1×10^4 cells/well) and cultured for 24 h. Cell proliferation was evaluated by the adding 10 μl of cell counting kit 8 into each well and measuring at optical density of 450 nm with a microplate reader.

Transwell Assay

Cell migration and invasion were tested with Transwell assay. For invasion assay, HeLa and Caski cells were pretreated with sevoflurane at various concentrations for 2 or 4 h. Then 200 μl of cell suspension containing 3×10^3 cells were seeded into the upper chamber coated with Matrigel (BD Biosciences, USA) in serum-free medium. The lower chambers were filled with 600 μl of medium supplemented with 10% fetal bovine serum. After being incubated for 20 h, the cells were fixed with 4% paraformaldehyde and stained with crystal violet. The cells that penetrated the Matrigel gel membrane into the lower chamber were counted using a microscopy. For migration assay, the upper chamber was not coated with Matrigel, whereas the other steps were identical as the invasion assay.

Small Interfering RNA Transfection

Histone deacetylase 6 small interfering RNA (sense strand, 5′-CUUCGAAGCGAAAUAUUAATT-3′; antisense strand, 5′-UUAAUAUUUCGCUUCGAAGTG-3′) and scramble small interfering RNA were obtained from Qiagen (Germany). A scramble small interfering RNA was synthesized for use as the control. The cells were cultured in 6-well plates (5×10^5 cells/well) reaching 30 to 50% confluence before transfection. The cells were transfected with histone deacetylase 6 small interfering RNA (10 μl) or scramble small interfering RNA (10 μl) using Lipofectamine RNAi Max (Invitrogen, USA) in serum-free medium. After incubation for 6 h, the medium was replaced by fresh medium containing 10% fetal bovine serum, and the cells were incubated for another 24 h. Then the cells were exposed to sevoflurane, followed by cell viability and Transwell assay as described above. Total protein lysates were harvested from cells and evaluated with Western blot analysis.

Protein Kinase B (AKT) and Extracellular Regulated Protein Kinases (ERK1/2) Inhibitor Treatment

HeLa or Caski cells were seeded into 6-well plates (8×10^5 cells/well). The cells were pretreated with the AKT inhibitor LY294002 (20 μM; Cell Signaling Technology) for 1 h prior to sevoflurane exposure. ERK1/2 inhibitor U0126 (10 μM; Cell Signaling Technology) was used to treat cells for 2 h, followed by sevoflurane treatment.

Western Blot Analysis

The proteins were extracted from cervical cancer cells using cell lysis buffer supplement with proteinase and phosphatase inhibitors. Then proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by polyvinylidene fluoride membrane transfer. The membranes were incubated overnight at 4°C with the primary antibodies as follows: anti–histone deacetylase 6, anti–acetylated α-tubulin , anti–α-tubulin, anti–phospho-AKT, anti-AKT, anti-ERK1/2, anti-phospho-ERK1/2, and anti–glyceraldehyde-3-phosphate dehydrogenase (1:1,000 dilution; Cell Signaling Technology). Protein bands were visualized by using enhanced chemiluminescence detection solution, and densitometric quantification was performed by ImageJ software.

Statistical Analysis

There were no missing data in the present study. No outliers were detected. The data were first tested for normality using the Shapiro–Wilk test and then were presented as means ± SD. The one-way ANOVA test was used in the present study for between subjects. When data were only compared to the controls, one-way ANOVA followed by Dunnett's T3 *post hoc* test was used. One-way ANOVA followed by Newman–Keuls *post hoc* comparison was used when data were compared between all groups. Unpaired *t* test was used to compare two independent samples. A two-sided *P* value of less than 0.05 was considered to be statistically significant. No statistical power calculation was conducted before the study. The sample size was based on the available data. All data were analyzed with Statistical Product and Service Solutions 17.0 statistical software.

Results

Sevoflurane Enhances Proliferation of HeLa Cells

To investigate the effect of sevoflurane on the proliferation of cervical cancer cells, HeLa and Caski cells were treated with different concentrations of sevoflurane, and cell viability was determined by cell counting kit 8 assay at different time points after sevoflurane exposure. Ki-67 immunostaining was also performed to assess the proliferative rate of cervical cancer cells. Sevoflurane did not affect the proliferation of Caski cells (fig. 1A); however, its exposure for either 2 or 4 h significantly increased the proliferation of HeLa cells in a time- and concentration-dependent manner to be 106 ± 2.7 and 107 ± 1.4% relative to the controls (n = 10; $P = 0.036$; $P = 0.022$) at 24 h after exposure and to be 106 ± 2.2% and 106 ± 1.7% relative to the controls (n =10; $P = 0.031$; $P = 0.023$) at the highest concentration of 3% sevoflurane studied, respectively (fig. 1B). Because 2% sevoflurane is approximately equivalent to one minimum alveolar concentration in adults, which is commonly used to maintain anesthesia concentration clinically, both cells were treated with 2% sevoflurane for 2 h and then cultured for 24 h for the follow-up experiments. Sevoflurane did not significantly affect Ki-67 immunofluorescence intensity in Caski cells ($P = 0.576$; fig. 1C), yet it significantly increased Ki-67 levels in HeLa cells to suggest higher proliferation ($P < 0.001$; fig. 1C).

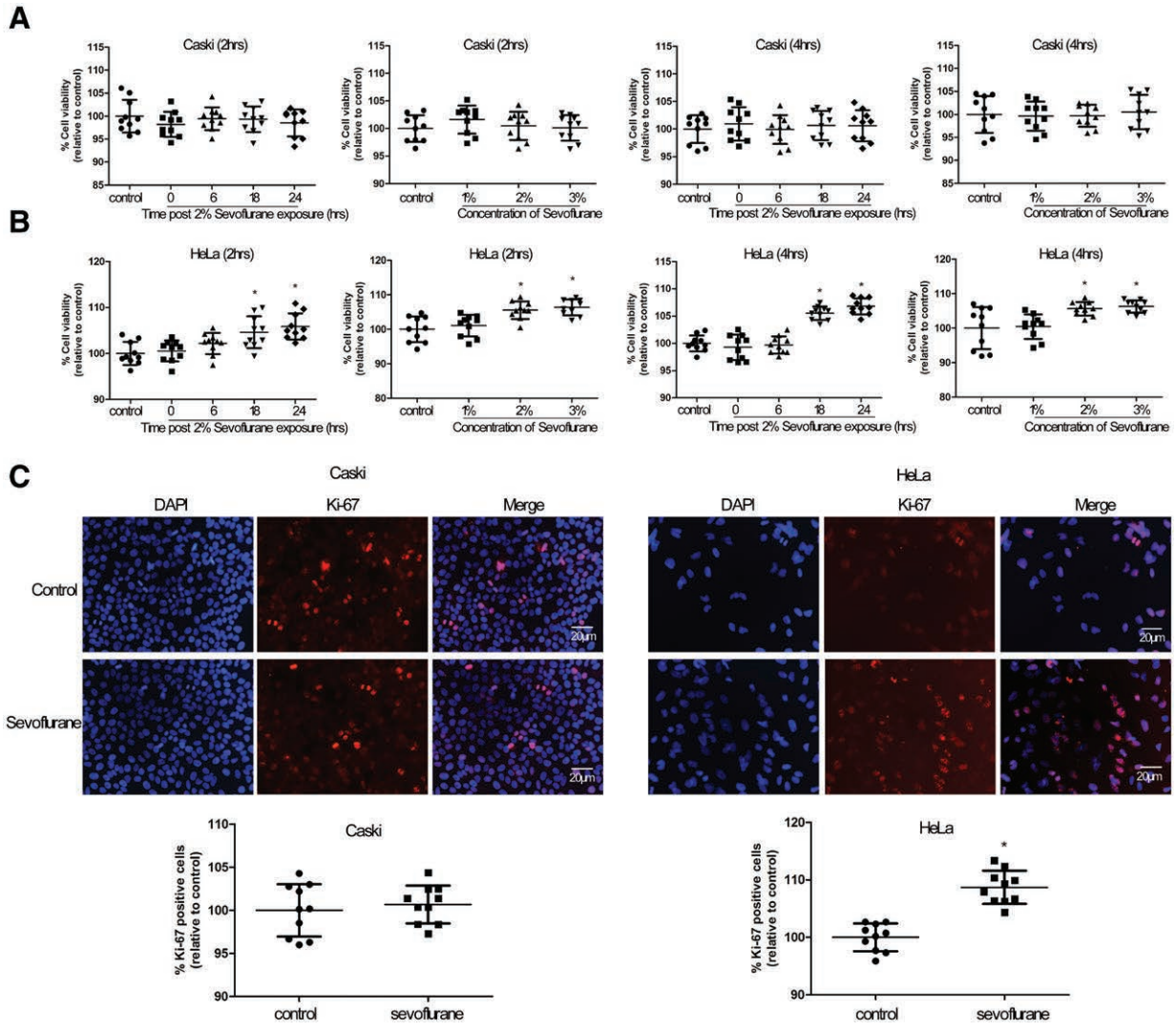

Fig. 1. Cervical cancer cell proliferation after sevoflurane exposure. Caski and HeLa cells were treated with or without different concentrations (1, 2, or 3%) of sevoflurane in 5% carbon dioxide balanced with air for 2 or 4 h, and cell viability assay with cell counting kit 8 (*A* and *B*) was performed at different time points (0, 6, 18, and 24 h) after gas exposure. Immunfluorescent analysis of Ki-67/4,6-diamidino-2-phenylindole (DAPI)–stained cells (*C*) was performed 24 h after gas exposure. The data are shown as means ± SD (n = 10). One-way ANOVA with Dunnett comparison (cell counting kit 8) and unpaired *t* test (Ki-67) were used. **P* = 0.022-0.036 *versus* control.

Sevoflurane Enhances Cell Migration and Invasion and Induces Cytoskeletal Rearrangement in Cervical Cancer Cells

To determine the effect of sevoflurane on cell migration, HeLa and Caski were exposed to different concentration of sevoflurane for 2 or 4 h, and the number of cells penetrating the membrane were assessed at 0, 2, 4, and 6 h after exposure by Transwell migration assay. Sevoflurane increased the migration of Caski cells at 4 h to 1.41 ± 0.10 and 6 h to 1.39 ± 0.08 after gas exposure for 2 h, relative to the controls ($F[4, 25] = 33.14$; $P < 0.001$; Supplemental Digital Content fig. 1, A and B, http://links.lww.com/ALN/C165). Furthermore, sevoflurane promoted migration of Caski cells at 2 h (1.28 ± 0.11), 4 h (1.59 ± 0.09), and 6 h (1.59 ± 0.12) compared with the control group (1.00 ± 0.09) after gas exposure for 4 h ($F[4, 25] = 32.26$; $P < 0.001$; Supplemental Digital Content fig. 1, A and B, http://links.lww.com/ALN/C165). Dose–response tests on migration of Caski cells showed that sevoflurane enhanced cell migration with a marked effect at 2 and 3% sevoflurane exposure for 2 h ($F[3, 20] = 62.27$; $P < 0.001$) and 4 h ($F[3, 20] = 118.00$; $P < 0.001$; Supplemental Digital Content fig. 1, A and B, http://links.lww.com/ALN/C165). Our results also showed enhanced migration of HeLa cells in a time- ($F[4, 25] = 193.30$, $P < 0.001$) and concentration-dependent

manner (F[3, 20] = 188.50; $P < 0.001$), with maximal increase at 6 h after exposure and at 3% sevoflurane for 4 h (Supplemental Digital Content fig. 1, C and D, http://links.lww.com/ALN/C165). Sevoflurane also increased the number of migrated HeLa cells at 4 h (1.75 ± 0.13) and 6 h (1.69 ± 0.22) after gas exposure for 2 h, compared with the control group (1.00 ± 0.10; F[4, 25] = 24.33; $P < 0.001$; Supplemental Digital Content fig. 1, C and D, http://links.lww.com/ALN/C165). In addition, sevoflurane enhanced migration of HeLa cells in a concentration-dependent manner for 2 h (F[3, 20] = 171.40, $P < 0.001$, Supplemental Digital Content fig. 1, C and D, http://links.lww.com/ALN/C165).

Using Transwell invasion assay, sevoflurane exposure significantly increased the number of invasive Caski cells with the highest increase to 1.63 ± 0.14 (relative to the control; F[3, 20] = 73.34; $P < 0.001$) at 3% sevoflurane and to 1.46 ± 0.13 (relative to the control) at 6 h after exposure (fig. 2, A and B; Supplemental Digital Content fig. 2, A and B, http://links.lww.com/ALN/C165). Additionally, 2% sevoflurane increased the number of HeLa cells that penetrated the membrane at 4 and 6 h after treatment (fig. 2, C and D). The highest increase (1.92 ± 0.12 relative to control; F[3, 20] = 180.80; $P < 0.001$) in invasion of HeLa cells was found at 3% sevoflurane treatment (fig. 2, C and D; Supplemental Digital Content fig. 2, C and D, http://links.lww.com/ALN/C165).

Cytoskeletal rearrangement was evaluated by immunofluorescent staining of the cellular microtubule components (*e.g.* fluorescein isothiocyanate–α-tubulin and rhodamine–F-actin). Sevoflurane caused extensive cytoskeletal rearrangement leading to more scattered cells of larger size (sevoflurane group *vs.* control group: 1.69 ± 0.21 *vs.* 1.00 ± 0.04; $P < 0.001$; Caski; 1.76 ± 0.13 *vs.* 1.00 ± 0.05; $P < 0.001$, HeLa; fig. 2E). Furthermore, membrane protrusions and membrane surface roughness were observed in the sevoflurane group.

Sevoflurane Induces the Expression of Histone Deacetylase 6 Protein in Cervical Cancer Cells

To determine the underlying molecular mechanism responsible for sevoflurane-induced cervical cancer cell migration and invasion, we investigated the expression of metastasis-related molecules by immunofluorescent analysis. No significant difference was found in the expression of matrix metalloproteinase 9 and E-cadherin proteins between the sevoflurane group and control group (data were not shown). To investigate the effects of sevoflurane treatment on histone deacetylase 6 protein levels, HeLa and Caski cells were exposed to 2% sevoflurane for 2 h followed by Western blot and immunofluorescent analysis at different time points after exposure. The fluorescence intensity of histone deacetylase 6 showed marked increase in both cell lines at 4 h after exposure relative to the control group (fig. 3A). Western blot mirrored the above findings, wherein sevofluane increased expression of histone deacetylase 6 in HeLa cells at 4 and 6 h and in Caski cells at 4 h after exposure (fig. 3B). Additionally, small interfering RNA knockdown of histone deacetylase 6 attenuated sevoflurane-induced migration and invasion of HeLa and Caski cells (figs. 3C and 4).

Sevoflurane Decreases the Acetylation of α-Tubulin in a Histone Deacetylase 6–dependent Manner

The effects of sevoflurane exposure on α-tubulin acetylation *in vitro* were explored. HeLa and Caski cells were treated with 2% sevoflurane for 2 h, and Western blot data revealed that the acetylation level of α-tubulin was decreased at 24 h after sevoflurane exposure in the both cells (fig. 5A). Immunofluorescent analysis also verified that sevoflurane decreased the level of acetylated α-tubulin when compared with the control group (Caski: 0.46 ± 0.06 *vs.* 1.00 ± 0.09, $P < 0.001$; HeLa: 0.51 ± 0.09 *vs.* 1.00 ± 0.13, $P < 0.001$; fig. 5B). On the other hand, small interfering RNA knockdown of histone deacetylase 6 restored the level of acetylated α-tubulin that was downregulated by sevoflurane treatment (fig. 5C).

Sevoflurane Increases Histone Deacetylase 6 *via* Phosphatidylinositide 3-Kinase/Protein Kinase B (AKT) and Extracellular Regulated Protein Kinases (ERK1/2) Pathway

The expression of histone deacetylase 6 may be increased through the mitogen–activated protein kinase or phosphatidylinositide 3-kinase/AKT pathway. To explore whether these pathways are involved in sevofluane-induced histone deacetylase 6 expression, HeLa and Caski were exposed to 2% sevoflurane for 2 h, and the levels of phosphorylated AKT and phosphorylated ERK1/2 were determined by Western blot at different time points after exposure. Sevoflurane led to significant increase in the levels of phosphorylated AKT and phosphorylated ERK1/2 in both cells (Supplemental Digital Content fig. 3A, http://links.lww.com/ALN/C165). However, sevoflurane did not have any effect on the expression levels of total AKT and total ERK1/2 ($P > 0.05$). When compared with the 2% sevoflurane only group, histone deacetylase 6 expression and phosphorylated AKT level were markedly decreased in the group receiving both phosphatidylinositide 3-kinase/AKT inhibitor (LY294002) and sevoflurane (Supplemental Digital Content fig. 3B, http://links.lww.com/ALN/C165). In addition, treatment with U0126, an ERK1/2 inhibitor, attenuated the up-regulation of phosphorylated ERK1/2 and histone deacetylase 6 after sevoflurane treatment (Supplemental Digital Content fig. 3C, http://links.lww.com/ALN/C165).

Discussion

The present study shows that sevoflurane can up-regulate cellular signaling pathways involving histone deacetylase 6,

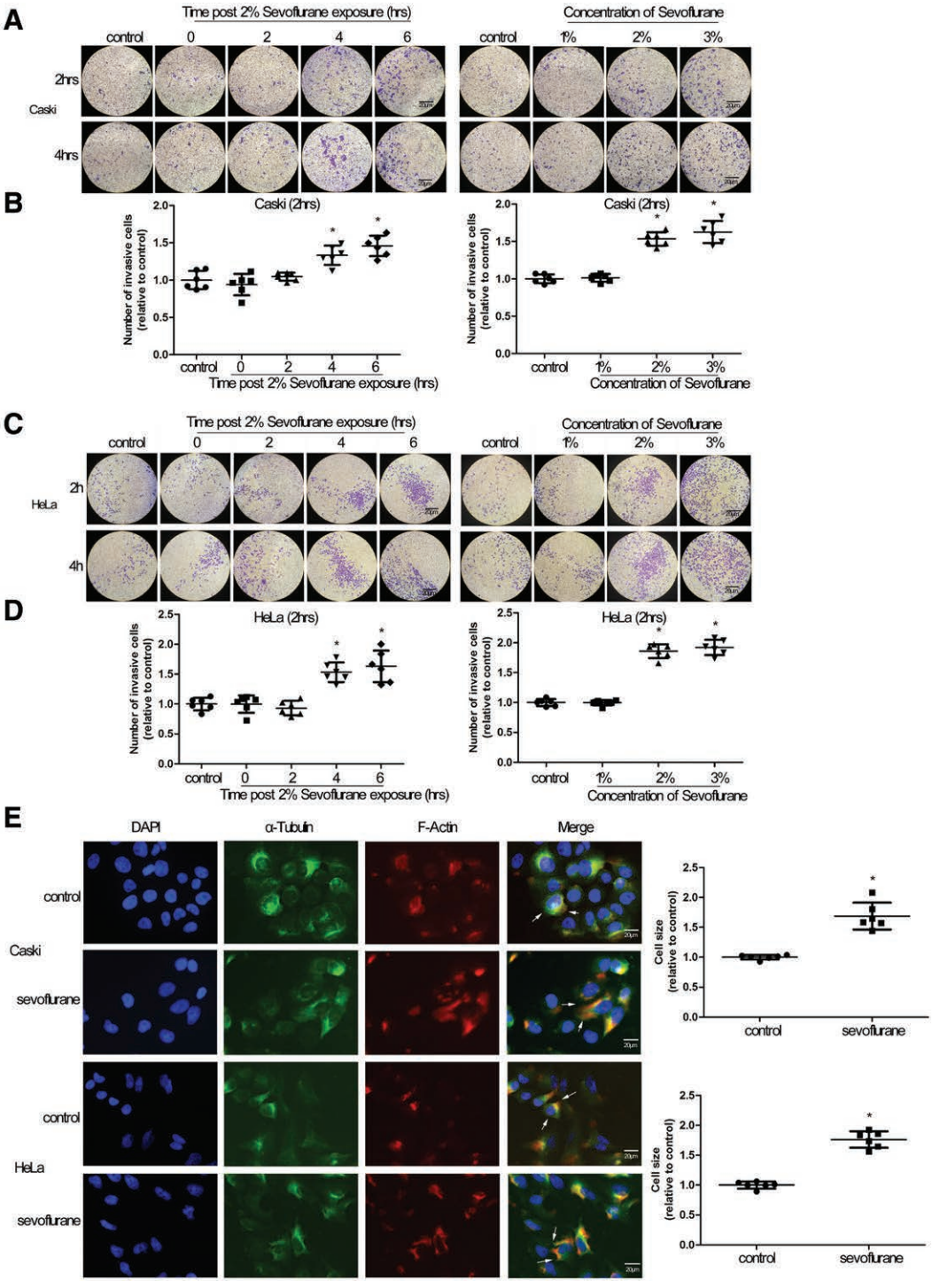

Fig. 2. Cervical cancer cell invasion after sevoflurane exposure. Caski cells were treated with or without different concentrations (1, 2, or 3%) of sevoflurane in 5% carbon dioxide balanced with air for 2 or 4 h, and Transwell assay (*A* and *B*) was performed at different time points (0, 2, 4, and 6 h) after gas exposure. HeLa cells were also exposed to sevoflurane for 2 or 4 h, and Transwell assay (*C* and *D*) was performed at 0, 2, 4, and 6 h after gas exposure, respectively. Immunofluorescent analysis of microstructural changes of Caski and HeLa cells after sevoflurane exposure was performed 4 h after 2% sevoflurane exposure for 2 h. Tetramethylrhodamine-5-(and 6)-isothiocyanate-phalloidin–F-actin and fluorescein isothiocyanate–α-tubulin were used for cell staining for visualization of the microtubular network (*E*). The cell area was measured by ImageJ software after sevoflurane exposure. The data are presented as means ± SD (n = 6). One-way ANOVA with Dunnett comparison (Transwell) and unpaired *t* test (cell size) were used. **P* < 0.001 *versus* control. DAPI, 4,6-diamidino-2-phenylindole.

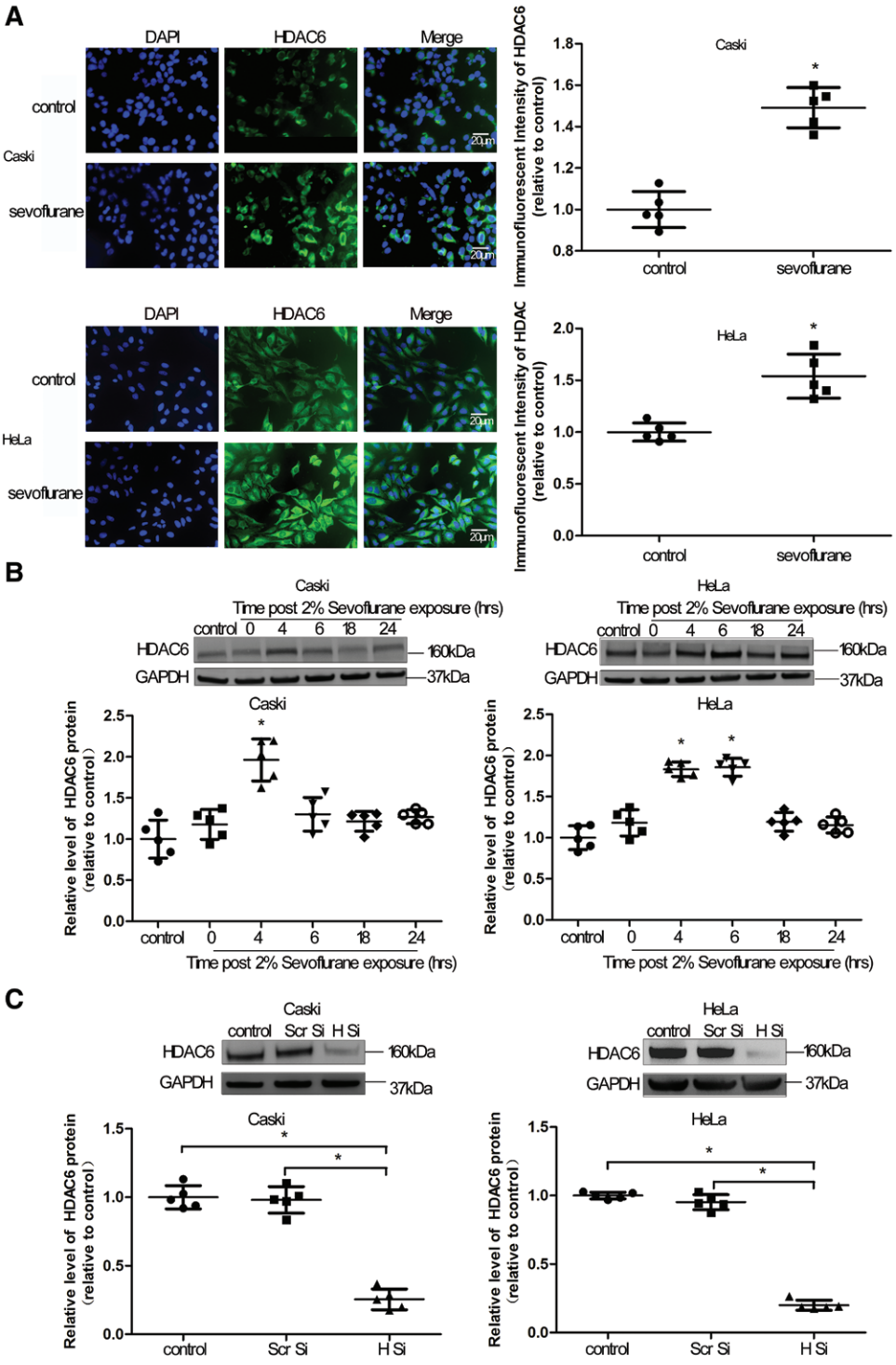

Fig. 3. Immunofluorescent and immunoblot analysis of metastasis-related protein changes after sevoflurane exposure. Caski and HeLa cells were treated with or without 2% sevoflurane and assessed with immunofluorescent analysis of histone deacetylase 6 (HDAC6; *A*) 4 h after gas exposure. Time course experiments were evaluated with Western blot against HDAC6 (*B*) at different time points (0, 4, 6, 18 and 24 h) after gas exposure. The expression of HDAC6 protein in cervical cancer cells transfected with HDAC6 small interfering RNA was analyzed by Western blot analysis (*C*). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level was used as internal controls. The data are presented as means ± SD (n = 5). Unpaired *t* test (immunofluorescence), one-way ANOVA with Dunnett test (Western blot), and one-way ANOVA with Newman–Keuls test (*C*) were used. **P* < 0.001 *versus* control or as present. DAPI, 4,6-diamidino-2-phenylindole; H Si, HDAC6 small interfering RNA; Scr Si, scramble small interfering RNA.

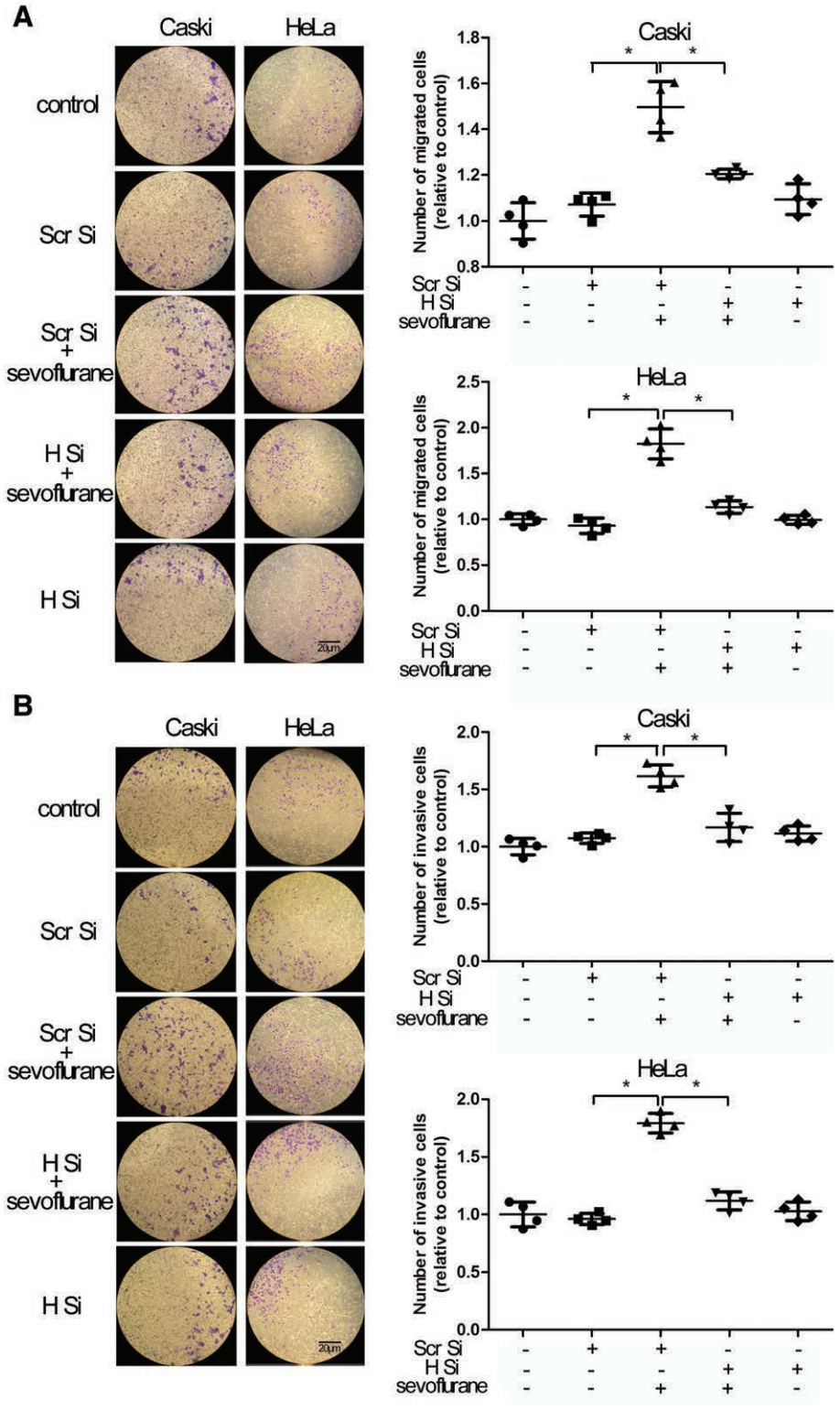

Fig. 4. Effects of histone deacetylase 6 on sevoflurane-induced migration and invasion in both Caski and HeLa cells. The cells were transfected with histone deacetylase 6 small interfering RNA or scramble small interfering RNA, and the cells were exposed to 2% sevoflurane for 2 h. Then the Transwell assay was performed 6 h after gas exposure for migration (*A*) and invasion (*B*) of Caski and HeLa cells. The data are presented as means ± SD (n = 4). One-way ANOVA with Newman–Keuls test was used. **P* < 0.001. H Si, histone deacetylase 6 small interfering RNA; Scr Si, scramble small interfering RNA.

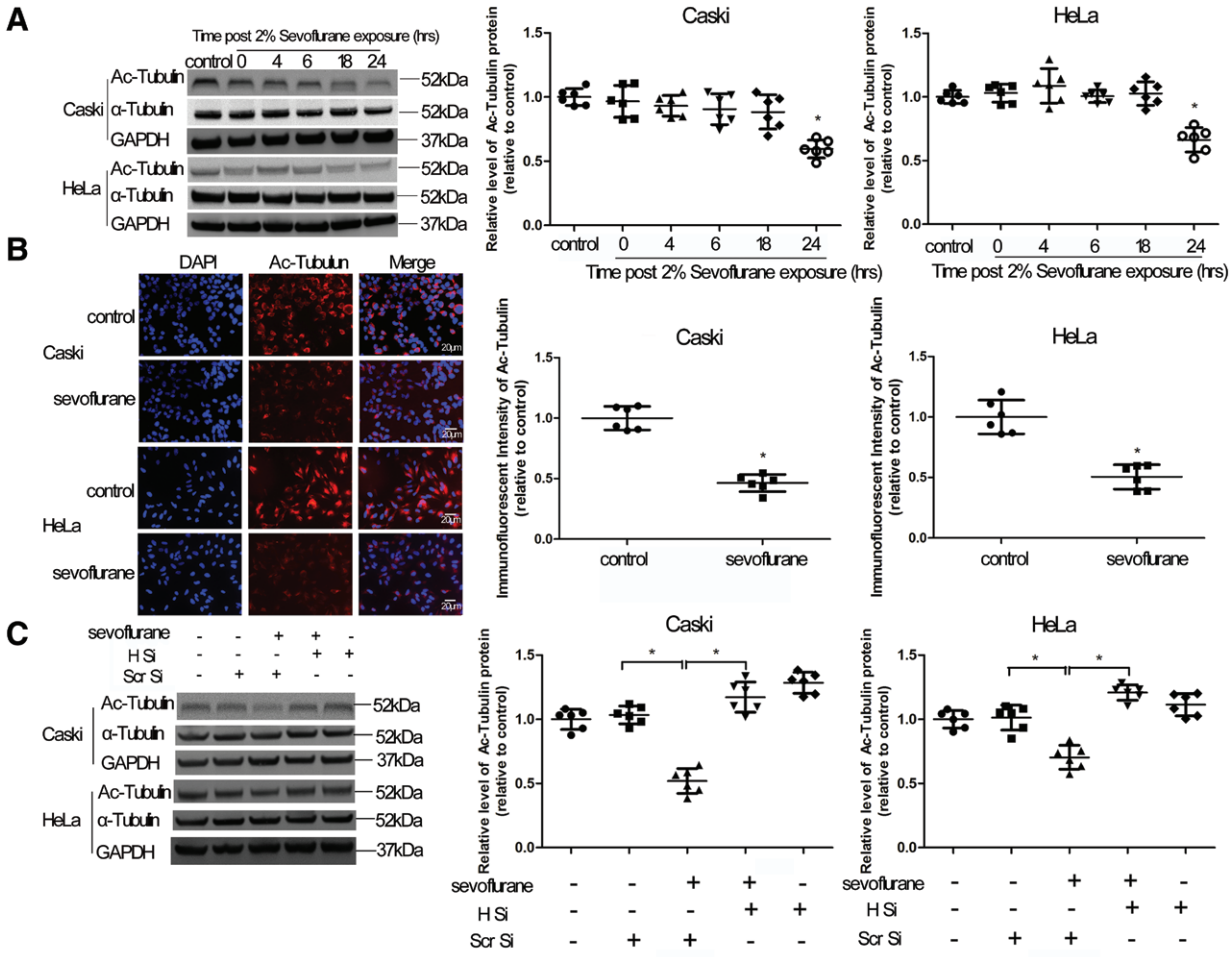

Fig. 5. Immunoblot and immunofluorescent analysis of histone deacetylase 6–dependent deacetylation of α-tubulin after sevoflurane exposure. Caski and HeLa cells were treated with or without 2% sevoflurane for 2 h, and Western blot against acetylated α-tubulin (Ac-Tubulin) was performed at different time points (0, 4, 6, 18, and 24 h) after gas exposure (*A*). Immunofluorescent analysis of acetylated α-tubulin was performed at 24 h after sevoflurane exposure (*B*). Effect of histone deacetylase 6 on deacetylation of acetylated α-tubulin detected by Western blot analysis is shown. The cells were transfected with histone deacetylase 6 small interfering RNA or scramble small interfering RNA, and the cells were exposed to 2% sevoflurane for 2 h. The expression of acetylated α-tubulin was assessed at 24 h after gas exposure (*C*). The data are presented as means ± SD (n = 6). One-way ANOVA with Dunnett's test (*A*), unpaired *t* test (*B*), and one-way ANOVA with Newman–Keuls test (*C*) were used. **P* < 0.001 *versus* control or as present. DAPI, 4,6-diamidino-2-phenylindole; H Si, histone deacetylase 6 small interfering RNA; Scr Si, scramble small interfering RNA.

which enhances malignant potential of cervical cancer cells. After exposure with clinically relevant concentrations of sevoflurane, it promoted cell proliferation, migration, and invasion and caused cytoskeletal rearrangement. Sevoflurane was found to increase histone deacetylase 6 expression *via* phosphatidylinositide 3-kinase/AKT and ERK1/2-dependent pathways, and sevoflurane-induced upregulation of histone deacetylase 6 promoted the migration and invasion of cervical cancer cells (fig. 6).

Abnormal proliferative capacity is one of the characteristic that distinguish cancer cells from normal cells.[19] The effects of sevoflurane on cancer cell proliferation is controversial. Previous studies reported a lack of effect of sevoflurane on the proliferation of U87MG glioma cells[20] and breast cancer cells.[21] However, sevoflurane was reported to exert the opposite effect in breast cancer cells when treated with sevoflurane in the liquid state.[6] These discrepancies may be attributed to the state-dependent effect of drug, whereby sevoflurane at liquid state or gaseous state could elicit different responses. In our previous study of prostate cancer cell line PC3, inhalational anesthetic isoflurane was demonstrated to promote cell proliferation through activation of hypoxia-inducible factor-1α pathway, which was suppressed by propofol treatment.[22] Our current results showed that sevoflurane had no effect on the proliferation of Caski cells. However, sevoflurane was found to

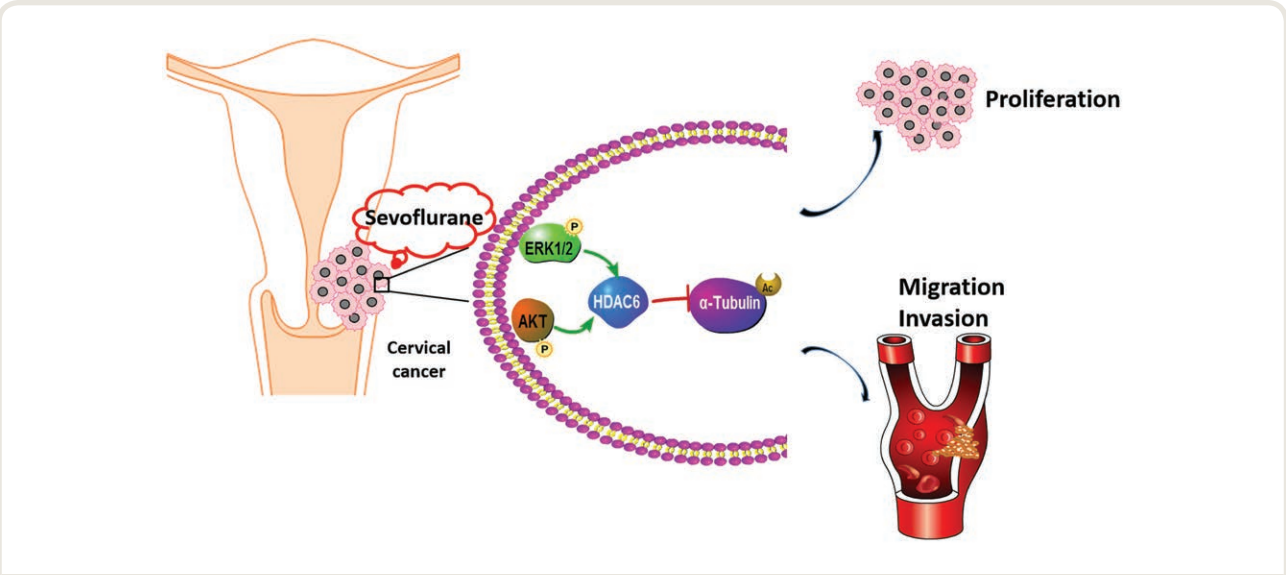

Fig. 6. Histone deacetylase 6 (HDAC6) up-regulation by sevolfurane and its downstream effects in the cervical cancer cells. Sevoflurane increases the expression of HDAC6 *via* the phosphatidylinositide 3-kinase/protein kinase B (AKT)/extracellular regulated protein kinases (ERK1/2) pathway. Subsequently, the acetylation of α-tubulin is decreased in an HDAC6-dependent manner. HDAC6 may, therefore, be responsible for sevoflurane-induced migration and invasion of cervical cancer cells. Ac, acetylated; P, phosphated.

increase the level of Ki-67 expression and enhance proliferation of HeLa cells in a concentration- and time-dependent manner. Because the pathologic types and HPV types of the two cancer cells are different (HeLa: HPV-18 positive; Caski: HPV-16 and -18 positive), it seems that the sensitivity of such two cancer cells to sevoflurane are also different. It indicates that HeLa cells are more susceptible to sevoflurane than Caski cells in view of cell proliferation.

Metastasis of cancer cells is a multistep process characterized by the interactions within the extracellular matrix, involving the adhesion molecules, decomposition of the matrix components, and migration of cancer cells, which requires cell motility.[23,24] Sevoflurane was shown to have no effect on the migration of MCF-7 and MDA-MB-231 breast cancer cells.[25] However, other groups have revealed that sevoflurane inhibited the invasion and migration of cancer cells such as colon cancer and glioma.[7,26] It was reported that sevoflurane inhibited migration of U251 glioma cells *via* up-regulating microRNA-637 and reducing the expression of phosphorylated AKT1 and total AKT1.[26] In contrast, we found that isoflurane enhanced angiogenesis and migration of ovarian cancer SK-OV3 cells *via* up-regulating of matrix metalloproteinase 2 and 9 expression.[27] In renal cell carcinoma RCC cells, isoflurane and sevoflurane promoted migration through upregulation of transforming growth factor-β or hypoxia-inducible factor-1α pathway.[9,19] In the present study, our data again showed that sevoflurane promoted migration and invasion of HeLa and Caski cells. Changes of cytoskeletal structure is a crucial step in the metastasis process, resulting in the spread of tumor cells and failure of therapy.[28] Microtubule regulate cell differentiation, locomotion, mechanics, and malignant transformation.[29] Cell motility is driven by the polymerization of actin monomers into F-actin, forming membrane protrusions.[24] We found more membrane protrusions in HeLa and Caski cells after sevoflurane exposure, suggesting that sevoflurane increases motility of cervical cancer cells. The reasons for such discrepancies between ours and others remains unknown but the longer anesthetic exposure time in previous studies may be one of the reasons.

Histone deacetylase 6 is primarily found in the cytoplasm and plays a crucial role in many biologic processes such as cell migration, invasion, proliferation, and apoptosis.[30] Histone deacetylase 6 deacetylates α-tubulin, increasing dynamics of microtubule to enhance cell movement and reduce cell adhesion, thus promoting cell invasion and migration.[31] It has been shown that histone deacetylase 6 promoted the invasion of renal carcinoma cells *via* decreasing acetylated α-tubulin.[31] In the present study, we showed that sevoflurane can increase histone deacetylase 6 expression in cervical cancer cells. We also demonstrated that knockdown of histone deacetylase 6 blocked sevoflurane-induced migration and invasion of HeLa and Caski cells, suggesting that sevoflurane can enhance migration and invasion of cervical cancer cells through, at least in part, histone deacetylase 6. Our data also revealed that sevoflurane decreased the acetylation of α-tubulin, and this effect was abolished by knockdown of histone deacetylase 6, suggesting that sevoflurane enhances migration and invasion of cervical cancer cells by histone deacetylase 6–mediated acetylation of α-tubulin. Furthermore, sevoflurane induced cell cytoskeletal rearrangement, and subsequently cell enlargement occurred. The mechanism underlying such changes may be due to the ability of histone deacetylase 6

to deacetylate cortactin that results in reduced binding of cortactin to actin, specifically F-actin, to bring about changes in cell motility.[32] Histone deacetylase 6 has been shown to targets heat shock protein 90, tubulin, and cortactin, thus regulating chaperone function, motility, and cell adhesion.[15] In this regard, sevoflurane-induced histone deacetylase 6 expression may modulate other interacting partners, such as heat shock protein 90, and cortactin involved in cancer progression, which warrants further investigation.

Interestingly, the phosphatidylinositide 3-kinase/AKT signaling pathway is commonly disrupted in cancers, with AKT as a key member of the pathway, playing a prominent role in many processes involved in carcinogenesis.[33] In the present study, it has been found that histone deacetylase 6 expression level rose in response to sevoflurane by activating phosphatidylinositide 3-kinase/AKT pathway in cervical cancer cells, and furthermore histone deacetylase 6 elevation was abolished with AKT inhibitor pretreatment. Isoflurane was reported to activate AKT pathway and increase calcium release from endoplasmic reticulum into cytosolic space *via* inositol triphosphate receptor,[34] suggesting that sevoflurane-induced inositol triphosphate receptor–dependent release of calcium from the endoplasmic reticulum may be very likely associated with the sevoflurane-induced AKT activation and upregulation of histone deacetylase 6 expression.

ERK1/2 is a component of the mitogen–activated protein kinase family, involved in the regulation of different cellular processes, including cell differentiation, proliferation, and cell motility. General anesthetic propofol was shown to suppress cancer migration and invasion *via* the ERK1/2 pathway.[35,36] Additionally, ERK1/2 is a known upstream regulator of histone deacetylase 6, whereby it phosphorylates histone deacetylase 6 at serine 1035 to induce cell migration.[14] It may be concluded that the importance of such a pathway in mediating the protumor effect in cervical cancer, wherein sevoflurane induced histone deacetylase 6 expression and ERK1/2 phosphorylation in HeLa and Caski cells, was reversible by ERK1/2 inhibition.

There are some limitations in our study. For example, immortalized cervical cancer cell lines were used in the current study. Hence, the biology of those cells is very likely not to be as freshly isolated as primary cancer cells. Therefore, cautions need to be taken for interpreting the data reported in the current study. Moreover, the present study was more focused on molecular mechanism of sevoflurane-induced cervical cancer cell malignancy. How sevoflurane affects cervical cancer cell in *in vivo* settings is unknown and warrants future study. Authentication was only done at the initial stage, and potential phenotype changes during experiments may occur. However, we have taken great cautions to avoid cross-contamination, and also their cellular morphology and behavior were closely monitored throughout the duration of study.

In conclusion, sevoflurane promotes cultured cancer cell proliferation and is responsible for an increase in cervical cancer cell migration and invasion mediated by the up-regulation of histone deacetylase 6 through the phosphatidylinositide 3-kinase/AKT and ERK1/2 pathway. Our *in vitro* study provides some mechanistic insights into how sevoflurane could directly modulate the invasive properties of cervical cancer cells. However, the conclusions of the present study were derived from pure cultured cancer cell *in vitro* work, which is far away from clinical setting, and warrant further study.

Research Support

Supported by the National Research Foundation of Health and Family Planning Commission–Medical Science (Beijing, China) and Technology Project of Zhejiang Province grant No. WKJ-ZJ-1528 (Hangzhou, China; to Dr. Zhu), Project of Wenzhou Science and Technology Bureau grant No. Y20150197 (Wenzhou, China; to Dr. Zhang), Research Fund for Lin He's Academician Workstation of New Medicine and Clinical Translation grant No. 19331202 (Wenzhou, China; to Dr. Zhang), and a British Oxygen Company chair grant (to Dr. Ma) from the Royal College of Anaesthetists (London, United Kingdom).

Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Ma: Imperial College London, Chelsea and Westminster Hospital, London, SW10 9NH, United Kingdom. d.ma@imperial.ac.uk. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

References

1. Parkhurst JO, Vulimiri M: Cervical cancer and the global health agenda: Insights from multiple policy-analysis frameworks. Glob Public Health 2013; 8:1093–108
2. Shen Y, Yang L, Wang Z: Treatment of early bulky cervical cancer with neoadjuvant paclitaxel, carboplatin and cisplatin prior to laparoscopical radical hysterectomy and pelvic lymphadenectomy. Oncol Lett 2012; 3:641–5
3. Zhang X, Pan C, Zhou L, Cai Z, Zhao S, Yu D: Knockdown of ST6Gal-I increases cisplatin sensitivity in cervical cancer cells. BMC Cancer 2016; 16:949
4. Tavare AN, Perry NJ, Benzonana LL, Takata M, Ma D: Cancer recurrence after surgery: Direct and indirect effects of anesthetic agents. Int J Cancer 2012; 130:1237–50

5. Elias KM, Kang S, Liu X, Horowitz NS, Berkowitz RS, Frendl G: Anesthetic selection and disease-free survival following optimal primary cytoreductive surgery for stage III epithelial ovarian cancer. Ann Surg Oncol 2015; 22:1341–8
6. Ecimovic P, McHugh B, Murray D, Doran P, Buggy DJ: Effects of sevoflurane on breast cancer cell function *in vitro*. Anticancer Res 2013; 33:4255–60
7. Müller-Edenborn B, Roth-Z'graggen B, Bartnicka K, Borgeat A, Hoos A, Borsig L, Beck-Schimmer B: Volatile anesthetics reduce invasion of colorectal cancer cells through down-regulation of matrix metalloproteinase-9. Anesthesiology 2012; 117:293–301
8. Liang H, Gu M, Yang C, Wang H, Wen X, Zhou Q: Sevoflurane inhibits invasion and migration of lung cancer cells by inactivating the p38 MAPK signaling pathway. J Anesth 2012; 26:381–92
9. Ciechanowicz S, Zhao H, Chen Q, Cui J, Mi E, Mi E, Lian Q, Ma D: Differential effects of sevoflurane on the metastatic potential and chemosensitivity of non–small-cell lung adenocarcinoma and renal cell carcinoma *in vitro*. Br J Anaesth 2018; 120:368–75
10. Haakenson J, Zhang X: HDAC6 and ovarian cancer. Int J Mol Sci 2013; 14:9514–35
11. Lee YS, Lim KH, Guo X, Kawaguchi Y, Gao Y, Barrientos T, Ordentlich P, Wang XF, Counter CM, Yao TP: The cytoplasmic deacetylase HDAC6 is required for efficient oncogenic tumorigenesis. Cancer Res 2008; 68:7561–9
12. Shan B, Yao TP, Nguyen HT, Zhuo Y, Levy DR, Klingsberg RC, Tao H, Palmer ML, Holder KN, Lasky JA: Requirement of HDAC6 for transforming growth factor-β1–induced epithelial–mesenchymal transition. J Biol Chem 2008; 283:21065–73
13. Wu H, Cheng XW, Hu L, Takeshita K, Hu C, Du Q, Li X, Zhu E, Huang Z, Yisireyili M, Zhao G, Piao L, Inoue A, Jiang H, Lei Y, Zhang X, Liu S, Dai Q, Kuzuya M, Shi GP, Murohara T: Cathepsin S activity controls injury-related vascular repair in mice via the TLR2-mediated p38MAPK and PI3K–Akt/p-HDAC6 signaling pathway. Arterioscler Thromb Vasc Biol 2016; 36:1549–57
14. Williams KA, Zhang M, Xiang S, Hu C, Wu JY, Zhang S, Ryan M, Cox AD, Der CJ, Fang B, Koomen J, Haura E, Bepler G, Nicosia SV, Matthias P, Wang C, Bai W, Zhang X: Extracellular signal–regulated kinase (ERK) phosphorylates histone deacetylase 6 (HDAC6) at serine 1035 to stimulate cell migration. J Biol Chem 2013; 288:33156–70
15. Batchu SN, Brijmohan AS, Advani A: The therapeutic hope for HDAC6 inhibitors in malignancy and chronic disease. Clin Sci (Lond) 2016; 130:987–1003
16. Qin HX, Cui HK, Pan Y, Yang J, Ren YF, Hua CH, Hua FF, Qiao YH: HDAC6 siRNA inhibits proliferation and induces apoptosis of HeLa cells and its related molecular mechanism. Asian Pac J Cancer Prev 2012; 13:3367–71
17. Sakuma T, Uzawa K, Onda T, Shiiba M, Yokoe H, Shibahara T, Tanzawa H: Aberrant expression of histone deacetylase 6 in oral squamous cell carcinoma. Int J Oncol 2006; 29:117–24
18. Xu G, Huang YL, Li PL, Guo HM, Han XP: Neuroprotective effects of artemisinin against isoflurane-induced cognitive impairments and neuronal cell death involve JNK/ERK1/2 signalling and improved hippocampal histone acetylation in neonatal rats. J Pharm Pharmacol 2017; 69:684–97
19. Benzonana LL, Perry NJ, Watts HR, Yang B, Perry IA, Coombes C, Takata M, Ma D: Isoflurane, a commonly used volatile anesthetic, enhances renal cancer growth and malignant potential via the hypoxia-inducible factor cellular signaling pathway *in vitro*. Anesthesiology 2013; 119:593–605
20. Hurmath FK, Mittal M, Ramaswamy P, Umamaheswara Rao GS, Dalavaikodihalli Nanjaiah N: Sevoflurane and thiopental preconditioning attenuates the migration and activity of MMP-2 in U87MG glioma cells. Neurochem Int 2016; 94:32–8
21. Liu J, Yang L, Guo X, Jin G, Wang Q, Lv D, Liu J, Chen Q, Song Q, Li B: Sevoflurane suppresses proliferation by upregulating microRNA-203 in breast cancer cells. Mol Med Rep 2018; 18:455–60
22. Huang H, Benzonana LL, Zhao H, Watts HR, Perry NJ, Bevan C, Brown R, Ma D: Prostate cancer cell malignancy via modulation of HIF-1α pathway with isoflurane and propofol alone and in combination. Br J Cancer 2014; 111:1338–49
23. Aznavoorian S, Murphy AN, Stetler-Stevenson WG, Liotta LA: Molecular aspects of tumor cell invasion and metastasis. Cancer 1993; 71:1368–83
24. Olson MF, Sahai E: The actin cytoskeleton in cancer cell motility. Clin Exp Metastasis 2009; 26:273–87
25. Ash SA, Valchev GI, Looney M, Ni Mhathuna A, Crowley PD, Gallagher HC, Buggy DJ: Xenon decreases cell migration and secretion of a pro-angiogenesis factor in breast adenocarcinoma cells: Comparison with sevoflurane. Br J Anaesth 2014; 113:i14–21
26. Yi W, Li D, Guo Y, Zhang Y, Huang B, Li X: Sevoflurane inhibits the migration and invasion of glioma cells by upregulating microRNA-637. Int J Mol Med 2016; 38:1857–63
27. Luo X, Zhao H, Hennah L, Ning J, Liu J, Tu H, Ma D: Impact of isoflurane on malignant capability of ovarian cancer *in vitro*. Br J Anaesth 2015; 114:831–9
28. Kedrin D, van Rheenen J, Hernandez L, Condeelis J, Segall JE: Cell motility and cytoskeletal regulation in invasion and metastasis. J Mammary Gland Biol Neoplasia 2007; 12:143–52
29. Suresh S: Biomechanics and biophysics of cancer cells. Acta Biomater 2007; 3:413–38
30. Valenzuela-Fernández A, Cabrero JR, Serrador JM, Sánchez-Madrid F: HDAC6: A key regulator of

cytoskeleton, cell migration and cell–cell interactions. Trends Cell Biol 2008; 18:291–7

31. Tran AD, Marmo TP, Salam AA, Che S, Finkelstein E, Kabarriti R, Xenias HS, Mazitschek R, Hubbert C, Kawaguchi Y, Sheetz MP, Yao TP, Bulinski JC: HDAC6 deacetylation of tubulin modulates dynamics of cellular adhesions. J Cell Sci 2007; 120:1469–79
32. Zhang X, Yuan Z, Zhang Y, Yong S, Salas-Burgos A, Koomen J, Olashaw N, Parsons JT, Yang XJ, Dent SR, Yao TP, Lane WS, Seto E: HDAC6 modulates cell motility by altering the acetylation level of cortactin. Mol Cell 2007; 27:197–213
33. Brown JS, Banerji U: Maximising the potential of AKT inhibitors as anti-cancer treatments. Pharmacol Ther 2017; 172:101–15
34. Bickler PE, Zhan X, Fahlman CS: Isoflurane preconditions hippocampal neurons against oxygen-glucose deprivation: Role of intracellular Ca^{2+} and mitogen-activated protein kinase signaling. Anesthesiology 2005; 103:532–9
35. Chen X, Wu Q, You L, Chen S, Zhu M, Miao C: Propofol attenuates pancreatic cancer malignant potential via inhibition of NMDA receptor. Eur J Pharmacol 2017; 795:150–9
36. Xu YB, Du QH, Zhang MY, Yun P, He CY: Propofol suppresses proliferation, invasion and angiogenesis by down-regulating ERK-VEGF/MMP-9 signaling in Eca-109 esophageal squamous cell carcinoma cells. Eur Rev Med Pharmacol Sci 2013; 17:2486–94