European Review for Medical and Pharmacological Sciences 2020; 24: 11883-11890

Sevoflurane induction alleviates the progression of gastric cancer by upregulating the miR-34a/TGIF2 axis

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Abstract. – OBJECTIVE: This study aims to elucidate how sevoflurane affects the malignant progression of gastric cancer (GC) and its pharmacological mechanism.

MATERIALS AND METHODS: Dose-dependent and time-dependent regulations of sevoflurane on proliferation inhibition rate in AGS and BGC-823 cells were examined, and thus the optimal dose and treatment time of sevoflurane on GC cells were selected. Subsequently, proliferative and migratory abilities in sevoflurane-induced AGS and BGC-823 cells (3.4% sevoflurane induction for 6 h) were detected by CCK-8 and transwell assay, respectively. After sevoflurane induction, relative levels of miR-34a and TGIF2 in GC cells were determined by qRT-PCR and Western blot. Regulatory effects of miR-34a on GC cell phenotypes were also assessed. Furthermore, the *in vivo* function of miR-34a in GC growth was explored by generating xenografted GC in nude mice.

RESULTS: Sevoflurane induction time-dependently and dose-dependently enhanced proliferation inhibition rate in AGS and BGC-823 cells. The proliferative and migratory abilities in GC cells induced with 3.4% sevoflurane for 6 h were markedly attenuated. sevoflurane induction upregulated miR-34a, but downregulated TGIF2 in GC cells. TGIF2 was negatively regulated by miR-34a. Notably, overexpression of miR-34a inhibited proliferative and migratory abilities in sevoflurane-induced GC cells, and knockdown of miR-34a yielded the opposite results. In nude mice with xenografted GC tissues, sevoflurane treatment markedly reduced tumorigenic ability, which was improved by knockdown of miR-34a.

CONCLUSIONS: Sevoflurane weakens proliferative and migratory abilities in GC by upregulating miR-34a and downregulating TGIF2.

Key Words:
Sevoflurane, TGIF2, Gastric cancer.

Introduction

Globally, gastric cancer (GC) is the third lethal cancer affecting 723,000 deaths each year[1,2]. Although medical technologies have been improved, the mortality of GC ranks in the high place because of atypical symptoms in the early phase, uncertain biological characteristics and genetic heterogeneity[2-4]. Currently, surgery and comprehensive strategies, including chemotherapy, radiotherapy and immune therapy are the preferred options for GC patients. Postoperative recurrence and metastasis, however, seriously restrict the efficacy of GC treatment[5,6]. Incomplete surgical resection, surgery-induced tumor cell infiltration to the circulating blood, and the release of neuroendocrine mediators due to surgical stress are all important factors for tumor recurrence and metastasis[7,8]. Moreover, stress response to anesthetics is a vital factor influencing the long-term prognosis in tumor patients[9,10].

Sevoflurane is an inhaled anesthetic widely used in surgeries and it has a strong anesthesia efficacy and high controllability[11,12]. Therefore, sevoflurane exerts an important role in general anesthesia, especially in the maintenance of anesthesia[12,13]. In addition to anesthesia, sevoflurane is able to protect cells, organs, and injuries[13]. Previous studies have shown the protective effects of sevoflurane on ischemia-reperfusion injury of important organs (i.e., heart, brain, lung, and kidney). It is capable of attenuating apoptosis of myocardial cells, neurons or renal tubular epithelial cells[9,13,14]. The protective or anti-damage effect of sevoflurane is related to the cell type, displaying cell specificity[12]. Recently, sevoflurane has been identified to promote the apoptosis of leukemia Jurkat T lymphocytes[15]. The malignant progres-

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sion in laryngeal cancer and colon cancer are reduced after sevoflurane treatment[16,17]. Sevoflurane is often used for surgical anesthesia, which is suitable for GC surgery that requires a long period of inhaled anesthesia[9,18].

Cancer growth is a complicated process involving multiple genes and steps. Uncontrolled proliferative and migratory potentials in cancer cells are vital reasons for carcinogenesis[19]. Very recently, miRNAs have been well concerned as cancer regulators[20]. This investigation examined the potential of sevoflurane on proliferation inhibition rate in GC cells, and its influence on the malignant progression of GC.

Materials and Methods

Cell Lines and Reagents

GC cell lines (AGS and BGC-823) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). They were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) at 37°C with 5% CO_2. In culture medium, 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin were added. Cell passage was conducted using 1×trypsin + EDTA (ethylenediaminetetraacetic acid) when cells were grown to 80-90% confluence.

Transfection

Cells were inoculated in 6-well plates and cultured to 70-80%. Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfection plasmids, including miR-34a mimic, miR-34a inhibitor, NC mimic and NC inhibitor, were constructed by GeneCopoeia. Transfected cells for 48 h were collected for use.

Cell Proliferation Assay

Cells were inoculated in a 96-well plate with 2.5×10^3 cells/well. At day 1, 2, 3 and 4, absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Molecular Technologies, Kumamoto, Japan) for plotting the viability curves.

Transwell Migration Assay

AGS cells (4×10^4) and BGC-823 cells (2×10^4) were suspended in 200 μL of serum-free medium and applied on the top of transwell chamber (Thermo Fisher Scientific, Waltham, MA, USA). In the bottom, 500 μL of complete medium was applied. After 24 h of incubation, cells in the bottom were fixed in methanol for 15 min, dyed with crystal violet for 30 min and counted using a microscope (Olympus, Tokyo, Japan).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNAs were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNA underwent qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). U6 was the internal reference. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. miR-34a: Forward: 5'-GGGTGGCAGTGTCTTAGCT-3', Reverse: 5'-GTGCAGGGTCCGAGGT-3'; U6: Forward: 5'-TCTGTGGAACCCTCCACTCT-3', Reverse: 5'-GCTTAGGATGCTGCTCC-3'.

Western Blot

Cells were lysed in radioimmunoprecipitation assay (RIPA) containing 1 μM phenylmethylsulfonyl fluoride (PMSF) on ice for 15 min (Beyotime, Shanghai, China), followed by centrifugation at 13000×rpm, 4°C for 15 min. The concentration of cellular protein was determined by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Protein samples with the adjusted same concentration were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (80 V for 40 min and 100 V for 60 min) and loaded on polyvinylidene difluoride (PVDF) membrane (200 V for 60 min) (Millipore, Billerica, MA, USA). The membrane was cut into small pieces according to the molecular size and blocked in 5% skim milk for 2 h. They were incubated with primary and secondary antibodies, followed by band exposure and grey value analyses.

In Vivo Xenograft Model

This investigation was approved by the Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine Animal Center. A total of 15 male nude mice with 8 weeks old were classified in Blank + NC inhibitor group, sevoflurane + NC inhibitor group and sevoflurane + miR-34a inhibitor group, respectively. Mice in the former two groups were subcutaneously administrated with AGS cells transfected with NC inhibitor in the armpit, and those in the latter group were ad-

ministrated with AGS cells transfected with miR-34a inhibitor. sevoflurane inhalation was performed in the latter two groups. Tumor size was recorded every 5 days. Thirty days later, mice were sacrificed for harvesting GC tissues and weighing. Tumor volume = (Tumor width^2×tumor length)/2.

Statistical Analysis

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between two groups were analyzed using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered as statistically significant.

Results

Sevoflurane Induction Inhibited Proliferative Potential in GC

AGS and BGC-823 cells were induced with 0%, 1.7%, 3.4% and 5.1% sevoflurane, respectively. CCK-8 data showed that proliferation inhibition rate was dose-dependently enhanced, which achieved the point of inflection at 3.4% (Figure 1A). Subsequently, GC cells were induced with 3.4% sevoflurane for 2 h, 4 h, 6 h and 8 h, respectively. As expected, proliferation inhibition rate was time-dependently elevated, and it yielded the point of inflection at 6 h (Figure 1B). It is indicated that high-dose, long-term induction of sevoflurane markedly suppressed proliferative ability in GC.

Sevoflurane Induction Attenuated Proliferative and Migratory Abilities in GC

AGS and BGC-823 cells were either induced with 3.4% sevoflurane for 6 h or not (blank control). Compared with blank control, sevoflurane induction markedly decreased viability and the number of migratory GC cells (Figure 2A, 2B).

Sevoflurane Induction Upregulated MiR-34a and Downregulated TGIF2

Of note, it is found miR-34a was markedly upregulated by sevoflurane induction in AGS and BGC-823 cells (Figure 3A). However, Western blot analysis showed that protein level of TGIF2 was downregulated in sevoflurane-induced GC cells (Figure 3B).

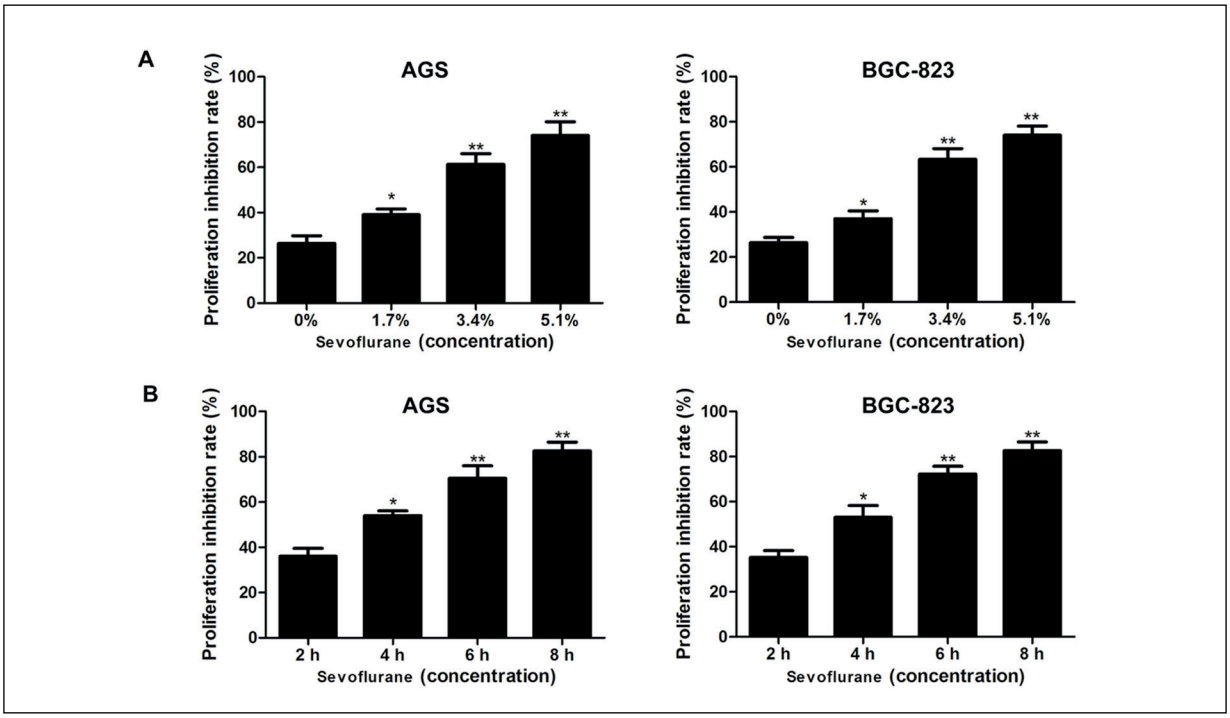

Figure 1. Sevoflurane induction inhibited proliferative potential in GC. **A,** Proliferation inhibition rate in AGS and BGC-823 cells induced with 0%, 1.7%, 3.4% and 5.1% sevoflurane. **B,** Proliferation inhibition rate in AGS and BGC-823 cells induced with sevoflurane for 2 h, 4 h, 6 h and 8 h. Data were expressed as mean±SD. *$p < 0.05$, **$p < 0.01$.

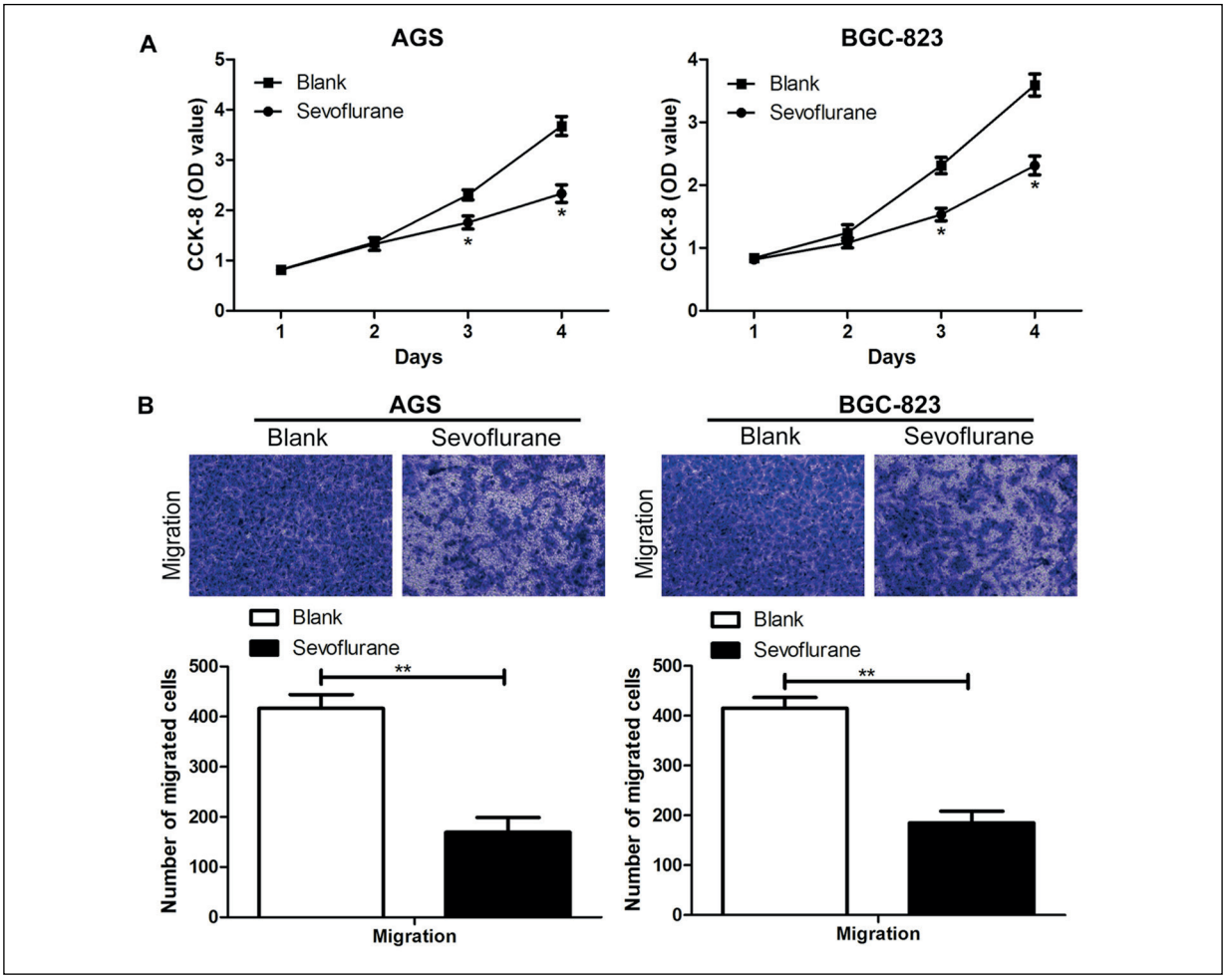

Figure 2. Sevoflurane induction attenuated proliferative and migratory abilities in GC. **A,** Viability in AGS and BGC-823 cells either treated with sevoflurane (3.4%, 6 h) or not. **B,** Migration in AGS and BGC-823 cells either treated with sevoflurane (3.4%, 6 h) or not (magnification: 200×). Data were expressed as mean±SD. *$p < 0.05$, **$p < 0.01$.

MiR-34a Attenuated Proliferative and Migratory Abilities in Sevoflurane-Induced GC Cells

To explore the involvement of miR-34a in mediating GC cell phenotypes, we constructed miR-34a mimic and inhibitor. Transfection of miR-34a mimic or miR-34a inhibitor in sevoflurane-induced AGS and BGC-823 cells effectively intervened miR-34a level, respectively (Figure 4A). Overexpression of miR-34a markedly reduced viability and migratory cell number in sevoflurane-induced AGS cells. On the contrary, knockdown of miR-34a in BGC-823 cells obtained the opposite results (Figure 4B, 4C). Western blot analyses showed that protein level of TGIF2 was negatively regulated by miR-34a in sevoflurane-induced GC cells (Figure 4D).

Sevoflurane Inhibited In Vivo Tumorigenesis of GC in Nude Mice

AGS cells were administrated in nude mice, followed by airway inhalation of sevoflurane. Compared with those in blank group, mice administrated with AGS cells transfected with NC inhibitor and inhaled with sevoflurane had smaller tumor volume and lower tumor weight (Figure 5A, 5B). *In vivo* knockdown of miR-34a in sevoflurane-induced mice markedly enhanced tumor volume and tumor weight of xenografted GC tissues. After mouse sacrifice, we collected the xenografted GC tissues for detecting protein level of TGIF2. Sevoflurane induction in nude mice bearing GC downregulated TGIF2 in tumor tissues. In addition, protein level of TGIF2 was higher in GC tissues harvested from sevoflurane-induced

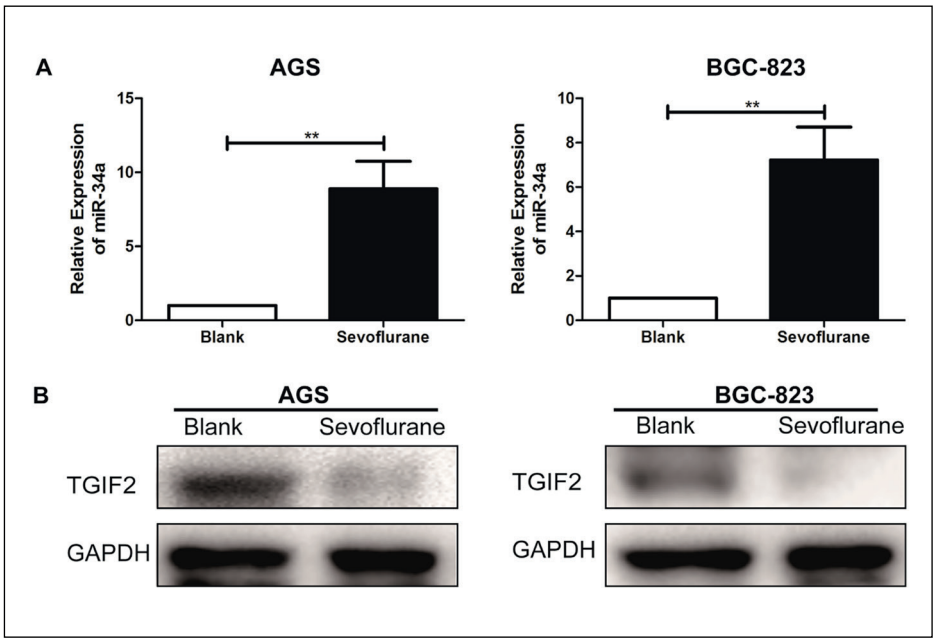

Figure 3. Sevoflurane induction upregulated miR-34a and downregulated TGIF2. **A,** MiR-34a level in AGS and BGC-823 cells either treated with sevoflurane (3.4%, 6 h) or not. **B,** Protein level of TGIF2 in AGS and BGC-823 cells either treated with sevoflurane (3.4%, 6 h) or not. Data were expressed as mean±SD. $^{**}p < 0.01$.

mice with *in vivo* knockdown of miR-34a than those inhaled with sevoflurane (Figure 5C). It is suggested that sevoflurane suppressed *in vivo* tumorigenesis of GC by upregulating the miR-34a/TGIF2 axis.

Discussion

Surgery is the main therapeutic strategy for solid tumors. Perioperative factors are closely linked to tumor recurrence and metastasis, as well as prognosis[5,6]. Notably, selection of anesthesia methods and drugs poses a certain impact on tumor prognosis[7-9]. Through retrospective analyses, local anesthesia has superior outcomes for breast cancer, prostate cancer, ovarian cancer and colorectal cancer surgeries than general anesthesia[8,9]. So far, the influence of surgery stress on human immunity and neuroendocrine has been considered to affect the outcomes of tumor patients[6,7]. For GC surgery, multiple types of anesthetics are required. Their influences on malignant phenotypes of GC, however, are rarely reported[9]. Sevoflurane is usually applied in anesthesia induction or maintenance for surgical resection of GC[10,11]. Its potential influence on biological characteristics of GC remains unclear. Through literature review and experimental experiences, here, AGS and BGC-823 cells were induced with sevoflurane (1.7%, 3.4% and 5.1%) for different time points (2h, 4h, 6h and 8h). As CCK-8 results demonstrated, sevoflurane induction dose-dependently and time-dependently inhibited proliferative ability in GC cells. Meanwhile, sevoflurane also weakened migratory ability in GC cells.

MiRNAs are able to regulate about one third of human genomes. During cancer progression, miRNAs serve as either oncogenes or tumor suppressors[19,20]. Through acting on single or multiple target genes, miRNAs are able to regulate GC cell functions[20]. Previous studies have shown that miR-34a participates in the progression and metastasis of GC[21]. Bioinformatic analysis uncovered the interaction between miR-34a and TGIF2. Our findings revealed that sevoflurane induction upregulated miR-34a and downregulated TGIF2 in GC cell lines. Notably, overexpression of miR-34a further weakened proliferative and migratory abilities in sevoflurane-induced GC cells. In nude mice with xenografted GC tissues, *in vivo* knockdown of miR-34a stimulated tumor growth of GC. Collectively, we have demonstrated that sevoflurane inhibited proliferative and migratory abilities in GC by activating the miR-34a/TGIF2 axis. Therefore, we suggested that sevoflurane should be considered for clinical application in subsequent years.

Conclusions

In brief, sevoflurane weakens proliferative and migratory abilities in GC by activating the miR-34a/TGIF2 axis.

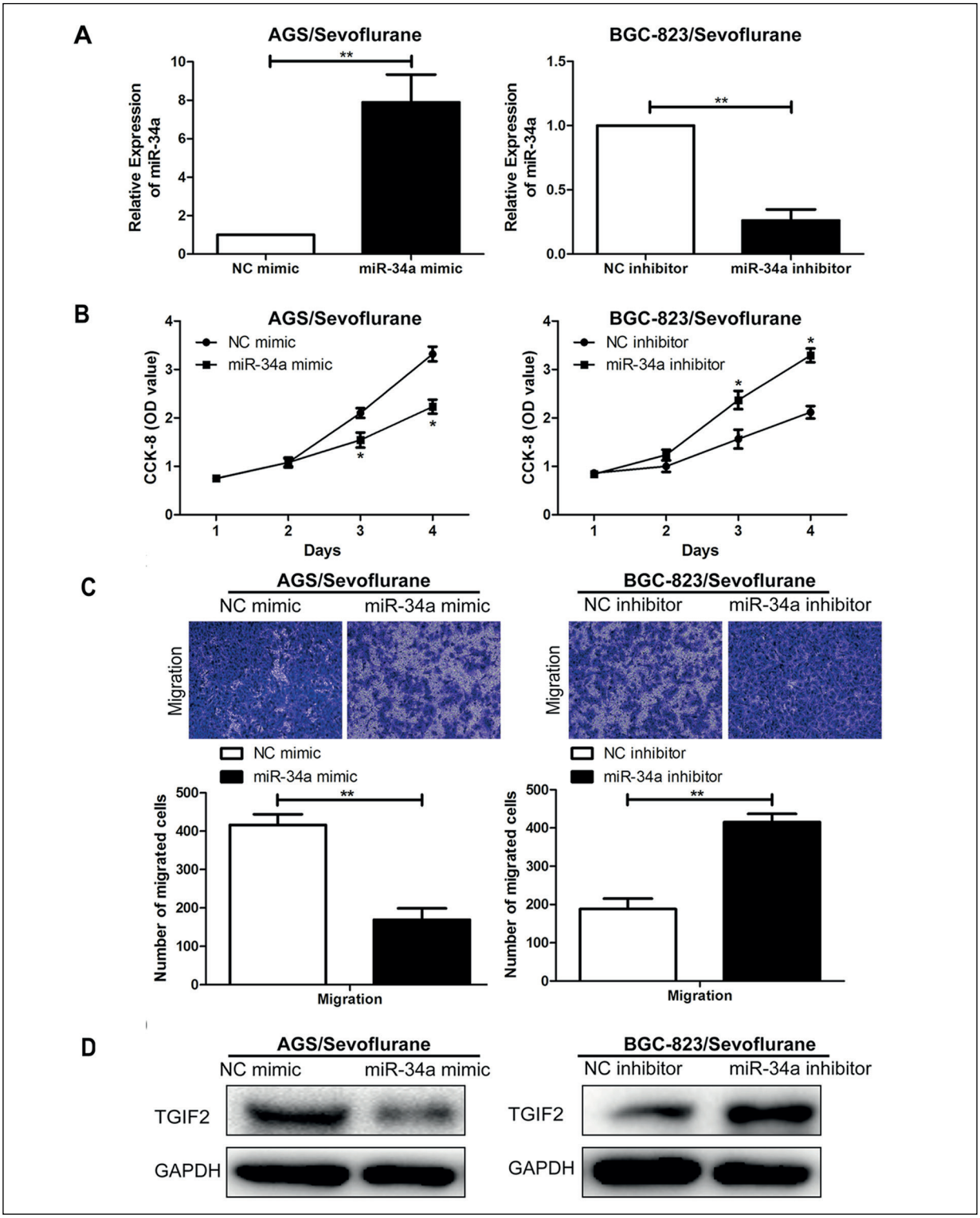

Figure 4. MiR-34a attenuated proliferative and migratory abilities in sevoflurane-induced GC cells. **A,** MiR-34a level in sevoflurane-induced AGS and BGC-823 cells transfected with miR-34a mimic or inhibitor, respectively. **B,** Viability in sevoflurane-induced AGS and BGC-823 cells transfected with miR-34a mimic or inhibitor, respectively. **C,** Migration in sevoflurane-induced AGS and BGC-823 cells transfected with miR-34a mimic or inhibitor, respectively (magnification: 200×). **D,** Protein level of TGIF2 in sevoflurane-induced AGS and BGC-823 cells transfected with miR-34a mimic or inhibitor, respectively. Data were expressed as mean±SD. $^{**}p < 0.01$.

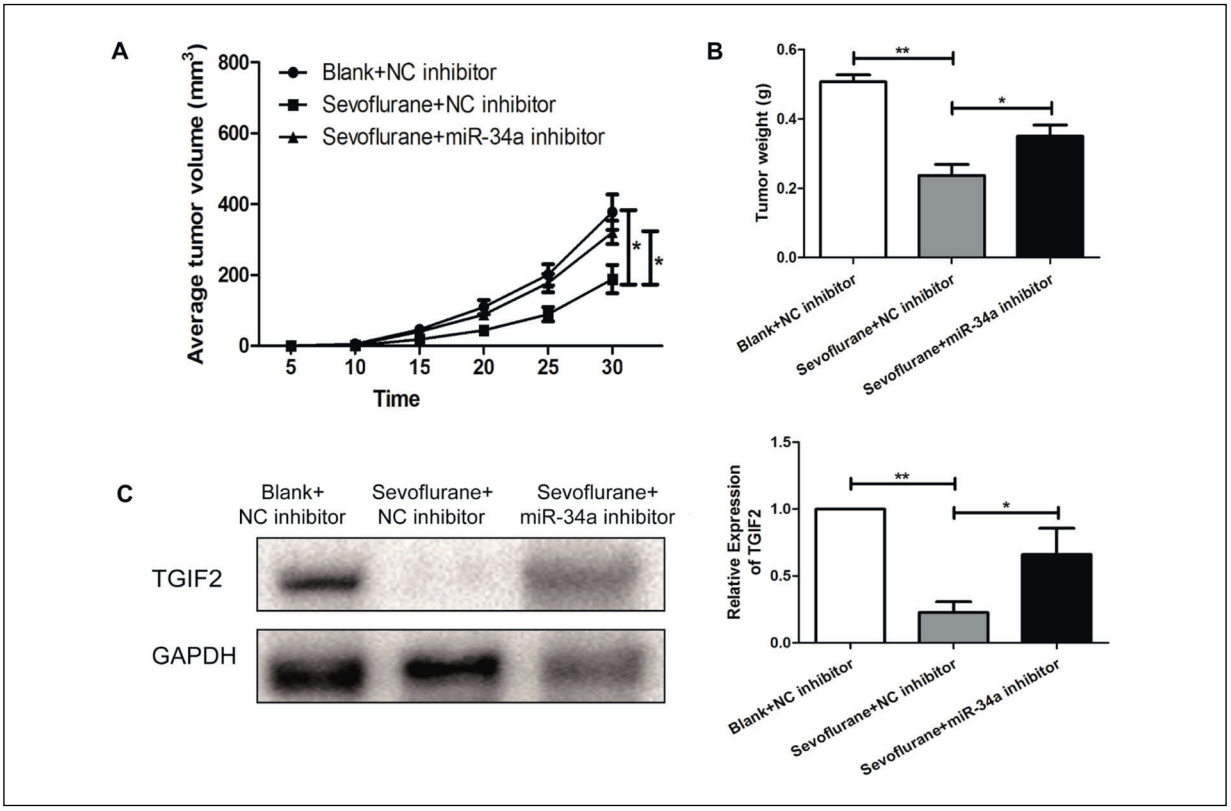

Figure 5. Sevoflurane inhibited *in vivo* tumorigenesis of GC in nude mice. **A,** Tumor volume in nude mice with xenografted GC and sevoflurane inhalation. **B,** Tumor weight in nude mice with xenografted GC and sevoflurane inhalation. **C,** Protein level of TGIF2 in GC tissues collected from nude mice with xenografted GC and sevoflurane inhalation. Data were expressed as mean±SD. $*p < 0.05$, $**p < 0.01$.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding Acknowledgements

General Project of Shanghai Health Committee, Project No.: 201840011.

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