Pathology & Oncology Research (2020) 26:947–954
https://doi.org/10.1007/s12253-019-00597-1

ORIGINAL ARTICLE

Sevoflurane Inhibits Glioma Cells Proliferation and Metastasis through miRNA-124-3p/ROCK1 Axis

Cao Gao[1] · Jiang Shen[1] · Zhi-Xiu Meng[1] · Xiao-Feng He[1]

Received: 27 July 2018 / Accepted: 15 January 2019 / Published online: 26 March 2019

Abstract
Malignant glioma is the most common primary malignancy in the brain. It is aggressive, highly invasive, and destructive. Studies have shown that sevoflurane can affect the invasion and migration of a variety of malignant tumors. However, its effects on human glioma cells and related mechanisms are not clear. Cultured U251 and U87 cells were pretreated with sevoflurane. The effect of sevoflurane on cell proliferation, migration, apoptosis and invasion ability were evaluated by MTT, wound healing assay, cell apoptosis and transwell assays, respectively. miRNA-124-3p and ROCK1 signaling pathway genes expression in sevoflurane treated cell lines was measured by quantitative real-time PCR (qRT-PCR) and western blotting analysis. The potential target genes of miRNA were predicted by online software. Luciferase reporter assay was employed to validate the direct targeting of ROCK1 by miRNA-124-3p. In present studies, sevoflurane inhibits glioma cells proliferation, invasion and migration. Additionally, inversely correlation between miR-124-3p and ROCK1 expression in sevoflurane treated glioma cells was observed. Furthermore, sevoflurane inhibits glioma cells proliferation, migration and invasion through miR-124-3p/ROCK1 axis. Taken together, our study revealed that sevoflurane can inhibit glioma cell proliferation, invasion and migration. Its mechanism may be related to the upregulation of miR-124-3p, which suppresses ROCK1 signaling pathway. The results of the study will help to understand the pharmacological effects of inhaled general anesthetics more comprehensively and help to provide an experimental basis for selecting more reasonable anesthetics for cancer patients.

Keywords Sevoflurane · Glioma · miRNA-124-3p · ROCK1

Introduction

Glioma is a common malignancy in the brain. It is aggressive, highly invasive, and destructive. Surgical resection of primary lesions combined with postoperative radio chemotherapy is one of the most effective ways to treat gliomas. But due to the limitations of brain tissue function and structure and the formation of tumor cell chemo-radiotherapy resistance, the vast majority of patients are refractory to recurrence, with a 5-years survival rate of less than 5% [1]. In recent years, there is increasing evidence that the use of perioperative anesthetic drugs will directly affect tumor cells and further influence the recurrence and metastasis of tumors and even the long-term survival time of patients [2]. Sevoflurane has the function of protecting brain function and is an inhalation anesthetic commonly used in craniocerebral surgery. Studies have shown that sevoflurane can affect the invasion and migration of a variety of malignant tumors [3]. However, its effects on human glioma cells and related mechanisms are not clear.

MicroRNAs (miRNAs) are a class of small regulatory RNAs that play a significant regulatory role in the progression of various cancers. MiRNAs are involved in various aspects of tumorigenesis, such as tumor cell proliferation, differentiation, metastasis, apoptosis, drug resistance, etc [4]. A number of miRNAs have been found to play a crucial role in the development and progression of gliomas, and miR-124 has been found to inhibit the proliferation, invasion, and metastasis of glioma cells [5]. Researchers used second-generation sequencing technology to find out that miR-124 is down-regulated in glioma tissue [6]. Studies have shown that miR-124-3p levels are significantly increased during sevoflurane-induced apoptosis of developing brain cells [7].

✉ Xiao-Feng He
hexiaofeng1567@163.com

[1] Department of Anesthesiology, The Third Affiliated Hospital of Soochow University, Changzhou 213002, People's Republic of China

Rho-associated coiled-coil containing protein kinase (ROCK) is a serine/threonine protein kinase and a downstream effector of RhoA. There are two isoforms of ROCK1 and ROCK2 in mammals. The ROCK1 protein was confirmed to be involved in tumor invasion and metastasis. Current research suggests that high expression of ROCK1 is associated with poorly differentiated tumors, lymphatic metastases, and short survival. Rho/ROCK pathway may be involved in the process of local infiltration and lymph node metastasis of tumor cells [8]. However, the molecular mechanisms involved in the regulation of ROCK1 have not yet been fully elucidated.

In the present study, we found sevoflurane inhibits the proliferation, invasion and migration abilities of glioma cells. The mechanism of this action is to up-regulate the expression of miR-124, thereby inhibiting the ROCK1 signaling pathway. The results of the study will help to understand the pharmacological effects of inhaled general anesthetics more comprehensively and help to provide an experimental basis for selecting more reasonable anesthetics for cancer patients.

Methods

Cell Culture and Transfection

Two different human glioma cell lines U251 and U87 were purchased from American type culture collection (ATCC). All of the cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen; USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen; USA). The sevoflurane-treated cells were cultured for 4 h in a gas atmosphere of 5% CO_2 and 90.9% O_2 and 4.1% sevoflurane. Cells were plated in 6-well plates at 1×10^6 cells per well and incubated at 37 °C for 24 h. Then transfected with 50 pmol inhibitor-miR-124-3p (GenePharmaCo, China) with Lipofectamine 2000 transfection reagent (Thermo; USA) as the protocol of manufacturer. Cells were collected 3 days post-transfection.

MTT Assay

Cells were seeded into 96-well plates (at density of 1×10^4 cells/well) and stained with MTT reagent (0.5 μg/μL) (Sigma; USA) for 4 h, following which the cells were washed twice with 1 × PBS and 150 μL DMSO (Sigma; USA) added. The absorbance at 490 nm (A_{490}) was measured after transfection.

Cell Invasion Assay

The transwell assay was performed to analysis the cell invasion ability. Briefly, cells were seeded into the upper chamber (1×10^6 cells/mL) and Dulbecco's modified Eagle's medium (DMSO) with 10% fetal bovine serum (PBS) was added in the lower chamber, and then incubated the chambers for 48 h at 37 °C. Stained the lower chamber with 0.1% crystal violet and counted the cell images under microscope.

Cell Migration Assay

For wound healing assay, U251 and U87 cells were seeded in 6-well plates with 1×10^6 cells/well and cultured till confluence. A wound was created by using a 10 μL pipette tip. Then cells were cultured with serum-free DMEM after washing with PBS buffer. The wound mark was measured after 24 h by using a Nikon light microscope.

Cell Apoptosis Assay

U251 and U87 cells were inoculated in 6-well plates with 1×10^6 cells/well and infected with tachyzoites (5×10^6 cells/mL) for 48 h. The cells were collected at 1000 rpm for 5 min and then stained by using Annexin V-FITC and propidium iodide (PI) kit (KeyGen Biotech, Nanjing, China) according to the protocol of manufacture. Finally, cell apoptosis was quantified by flow cytometry on Beckman Coulter flow cytometer (Becton Dickinson; USA).

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

The total RNA was extracted following the manufacturer's instruction of Trizol Reagent (Life technologies, Carlsbad, CA; USA). The quantitation of RNA was measured by NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE; USA). The primers for qRT-PCR were designed by Primer 5.0. GAPDH was selected as an internal control and relative number of transcripts were quantitated by $2^{-\Delta\Delta Ct}$ method.

Luciferase Reporter Assay

The pmirGLO vector was used in this study and the recombinant vector was transfected into U251 cells with miR-124 mimics or NC control, respectively. The miR-124-3p binding sites in ROCK1 were predicted by TargetScan(http://www.targetscan.org/vert_72/). The pmirGLO-ROCK1 or pmirGLO-mut-ROCK1 was transfected into U251 cells with miR-124 mimics or NC control. A Dual-Luciferase Reporter Assay kit (Promega; USA) was used to measure the luciferase activities as the protocol of manufacturer.

Western Blotting Assay

Protein was extracted with RIPA lysis buffer and 30 μg of isolated protein was separated on SDS-PAGE, then transferred

to PVDF membrane (Millipore; USA). The PVDF membrane was blocked with 5% skim milk and immunoblotted with the primary antibodies (rabbit anti-ROCK1 diluted 1:1000; rabbit anti- MMP2 diluted 1:1000; rabbit anti-MMP9 diluted 1:1000) at 4 °C overnight. Then incubated the membrane with anti-rabbit IgG antibody (ZSGB-BIO; China). Visualization was performed by using ECL assay (Millipore; USA).

Statistical Analysis

All of the experiments were performed at least three replicates. Comparisons of different group parameters were performed by using One-way ANOVA or Student's *t* test. A value of *P* less than 0.05 was considered to indicate a statistically significant difference. * and ** means $P < 0.05$ and $P < 0.01$ respectively. Statistical analysis was performed by using the SPSS statistical software package (Version 22.0 SPSS, Chicago, IL; USA).

Results

Sevoflurane Inhibits Glioma Cells Proliferation, Invasion and Migration

To investigate whether sevoflurane can affect cancer progression, U251 and U87 were treated with sevoflurane for 0 h, 24 h, 48 h and 72 h. MTT assay showed that sevoflurane significantly inhibited U251 and U87 cells proliferation after 72 h in culture (Fig. 1a). Moreover, sevoflurane significantly

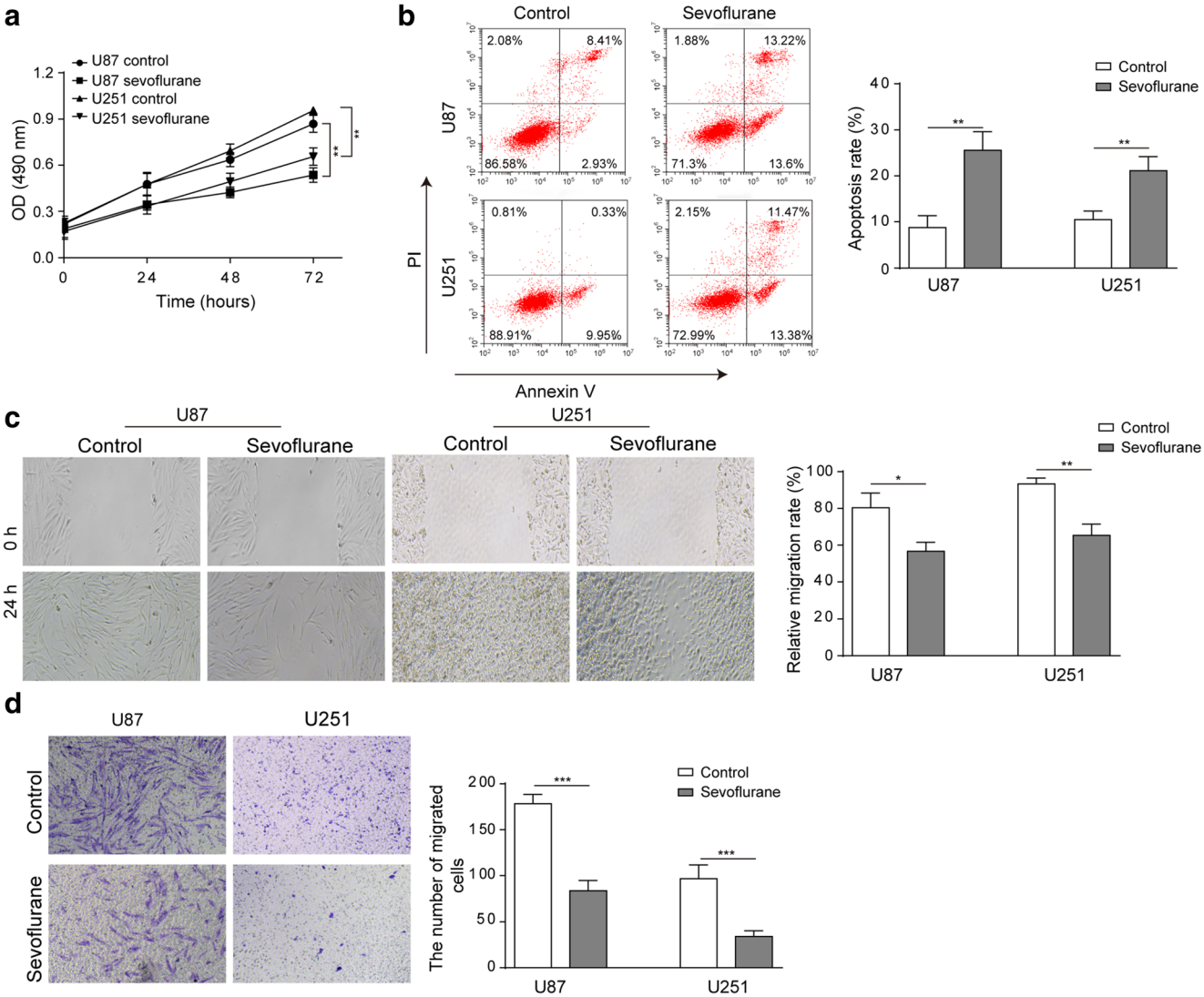

Fig. 1 Sevoflurane inhibits glioma cell proliferation and promotes apoptosis. a MTT assays were used to evaluate the effect of sevoflurane on proliferation ability of U251 and U87 cells. **b**, **c** Flow cytometry was used to evaluate the effect of sevoflurane on apoptosis ability of U251 and U87 cells. **d**, **e** Wound healing assays were used to evaluate the effect of sevoflurane on migration ability of U251 and U87 cells. **f**, **g** Transwell assays were used to evaluate the effect of sevoflurane on invasion ability of U251 and U87 cells. $^{**}P < 0.01$, $^{*}P < 0.05$, $n = 3$

enhanced the apoptosis rate of U251 and U87 cells (Fig. 1b, c). As predicted, sevoflurane significantly decreased the migration ability of U251 and U87 cells as shown in the wound healing assay (Fig. 1d, e). Transwell assay on U251 and U87 cells treated with sevoflurane showed that sevoflurane significantly attenuated the migration ability of the cells (Fig. 1f, g). Taken together, these results showed that sevoflurane could inhibit glioma cells proliferation, migration and enhanced its apoptosis.

Inversely Correlation between miR-124-3p and ROCK1 Expression in Sevoflurane Treated Glioma Cells

To evaluate the molecular mechanism by which sevoflurane alters glioma cells proliferation, invasion and migration, the mRNA or protein expression levels of miR-124-3p and ROCK1 were detected, respectively. The relative expression levels of miR-124-3p were measured by qRT-PCR. The results showed that the expression levels of miR-124-3p were significantly up-regulated in sevoflurane treated glioma cells compared with the control group (Fig. 2a). Furthermore, Western blotting assay was performed to measure ROCK1 and its downstream genes MMP-2 and MMP-9 protein expression levels. The results showed that the protein expression levels of ROCK1 and its downstream genes MMP-2 and MMP-9 were significantly down-regulated in sevoflurane treated glioma cells compared with the control group, and the trend of mRNA expression level was consistent with that of protein expression level (Fig. 2b, c). These findings strongly indicate that there is an inversely correlation between miR-124-3p and ROCK1 expression in sevoflurane treated glioma cells.

Sevoflurane Inhibits Glioma Cells Proliferation, Migration and Invasion through miR-124-3p

To further characterize whether miR-124-3p can affect glioma cells proliferation, migration and invasion after sevoflurane treated, miR-124-3p was knocked down in sevoflurane treated U251 and U87 cells, and the cells were examined by MTT, wound healing and transwell assays respectively. As we expected, cells transfected miR-124-3p inhibitor after sevoflurane treated showed dramatically higher proliferation capabilities (Fig. 3a), lower apoptosis rates (Fig. 3b, c), higher migration capabilities (Fig. 3d, e) and higher invasion capabilities (Fig. 3f, g) compared to cells only treated with sevoflurane. Thus, knockdown of miR-124-3p expression can rescue the sevoflurane-induced suppression of cell proliferation as well as sevoflurane-induced cell migration and invasion. These results demonstrate that up-regulation of miR-124-3p is one mechanism by which sevoflurane exerts its tumor suppressor functions.

Sevoflurane Suppresses the ROCK Signaling Pathway through Up-Regulate miR-124-3p

According to the above results, sevoflurane suppresses ROCK signaling pathway in glioma cells, we next investigated whether sevoflurane controls the ROCK signaling pathway via ROCK1-miR-124-3p axis in glioma cells. The level of miR-124-3p were decreased in both U251 and U87 cells transfected with miR-124-3p inhibitor after sevoflurane treated, compared with cells only treated with sevoflurane (Fig. 4a). The glioma cells transfected with miR-124-3p inhibitor after sevoflurane treated showed dramatically higher ROCK1 and its downstream genes MMP-2 and MMP-9 expression compared to cells only treated with sevoflurane (Fig. 4b, c). These findings demonstrated that sevoflurane suppressed the ROCK signaling pathway through up-regulate miR-124-3p.

MiR-124-3p Directly Targets ROCK1

The previous evidences show that ROCK1, a class of the target genes of miRNAs, is an oncogene in malignant glioma. However, the co-expression of miR-124-3p and ROCK1 in malignant glioma remains unclear. To test this, we constructed

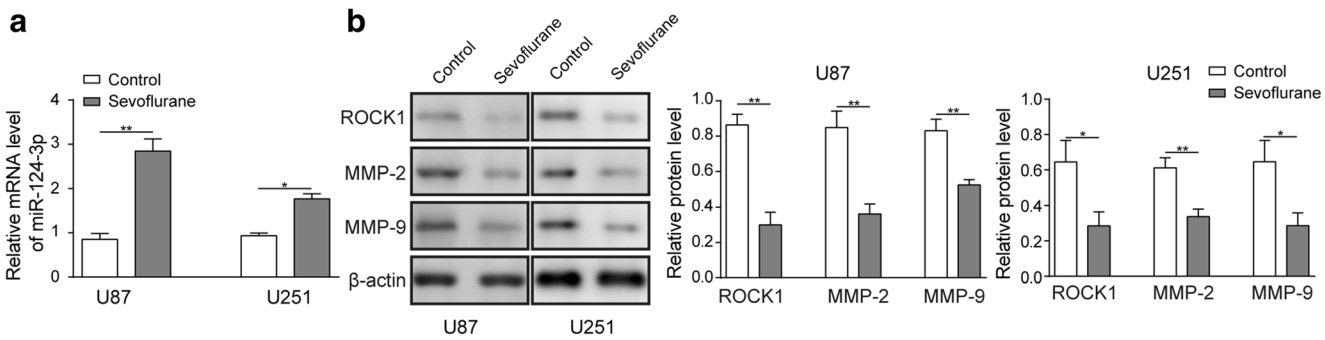

Fig. 2 Inversely correlation between miR-124-3p and ROCK1 expression in sevoflurane treated glioma cells. a qRT-PCR analysis of relative miR-124-3p expression levels in sevoflurane treated U251 and U87 cells. **b** Western blot analysis of the expression levels of ROCK1 protein and its downstream protein in U251 and U87 cells treated with sevoflurane. **c** qRT-PCR analysis of ROCK1 and its downstream gene mRNA levels in sevoflurane treated U251 and U87 cells. $^{**}P < 0.01$, $^{*}P < 0.05$, $n = 3$

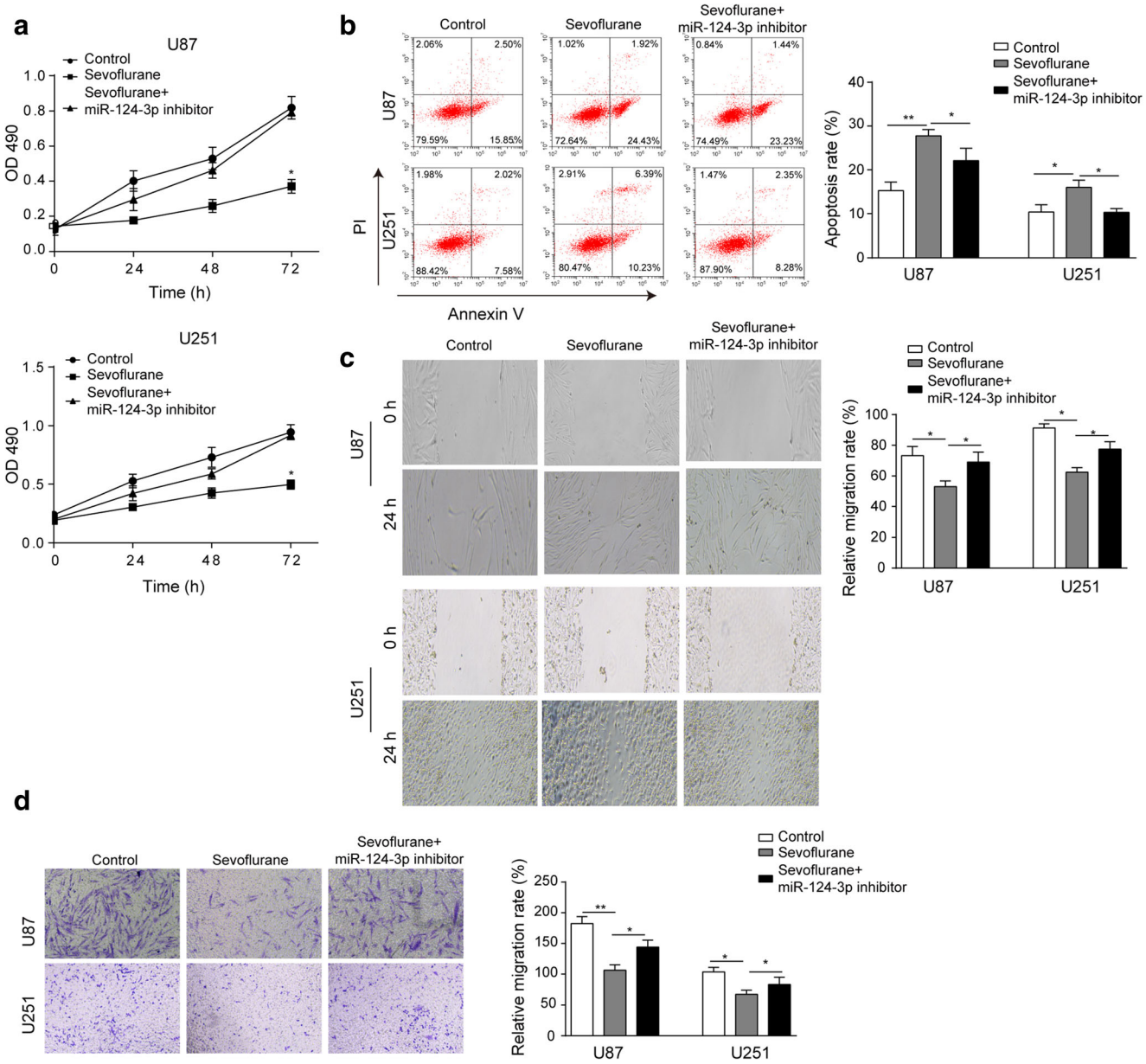

Fig. 3 Sevoflurane inhibits glioma cells proliferation, migration and invasion through miR-124-3p. a MTT assays were used to evaluate the effect of miR-124-3p inhibitor on proliferation ability of sevoflurane treated U251 and U87 cells. **b**, **c** Flow cytometry was used to measure the effect of miR-124-3p inhibitor on apoptosis ability of sevoflurane treated cells. **d**, **e** Wound healing assays were used to evaluate the effect of miR-124-3p inhibitor on migration ability of sevoflurane treated U251 and U87 cells. **f**, **g** Transwell assays were used to evaluate the effect of miR-124-3p inhibitor on invasion ability of sevoflurane treated U251 and U87 cells. $^{**}P < 0.01$, $^{*}P < 0.05$, $n = 3$

luciferase reporters pmirGLO-ROCK1 and pmirGLO-mut-ROCK1. The predicted wild-type binding site of miR-124-3p and the corresponding sequence of mutation were shown in Fig. 5a. We performed a luciferase reporter assay to illuminate that ROCK1 3'-UTR could complementary pair with miR-124-3p. The co-transfection of miR-124-3p mimics and pmirGLO-ROCK1 resulted in 36% luciferase activity decrease in glioma cells (Fig. 5b). Furthermore, the point mutations (pmirGLO-mut-ROCK1) were created by changing the first 2–8 bases to complementary sequence at ROCK1 3'-UTR to eliminate the binding site of miR-124-3p. However, there is no influence on the activity of luciferase (Fig. 5b). These data demonstrate that miR-124-3p directly target the 3′-UTR of ROCK1 gene.

Discussion

Glioma is a common tumor of the central nervous system, and the incidence rate accounts for about 40% of all central nervous system tumors. Surgical resection of primary lesions combined with postoperative radio chemotherapy is one of

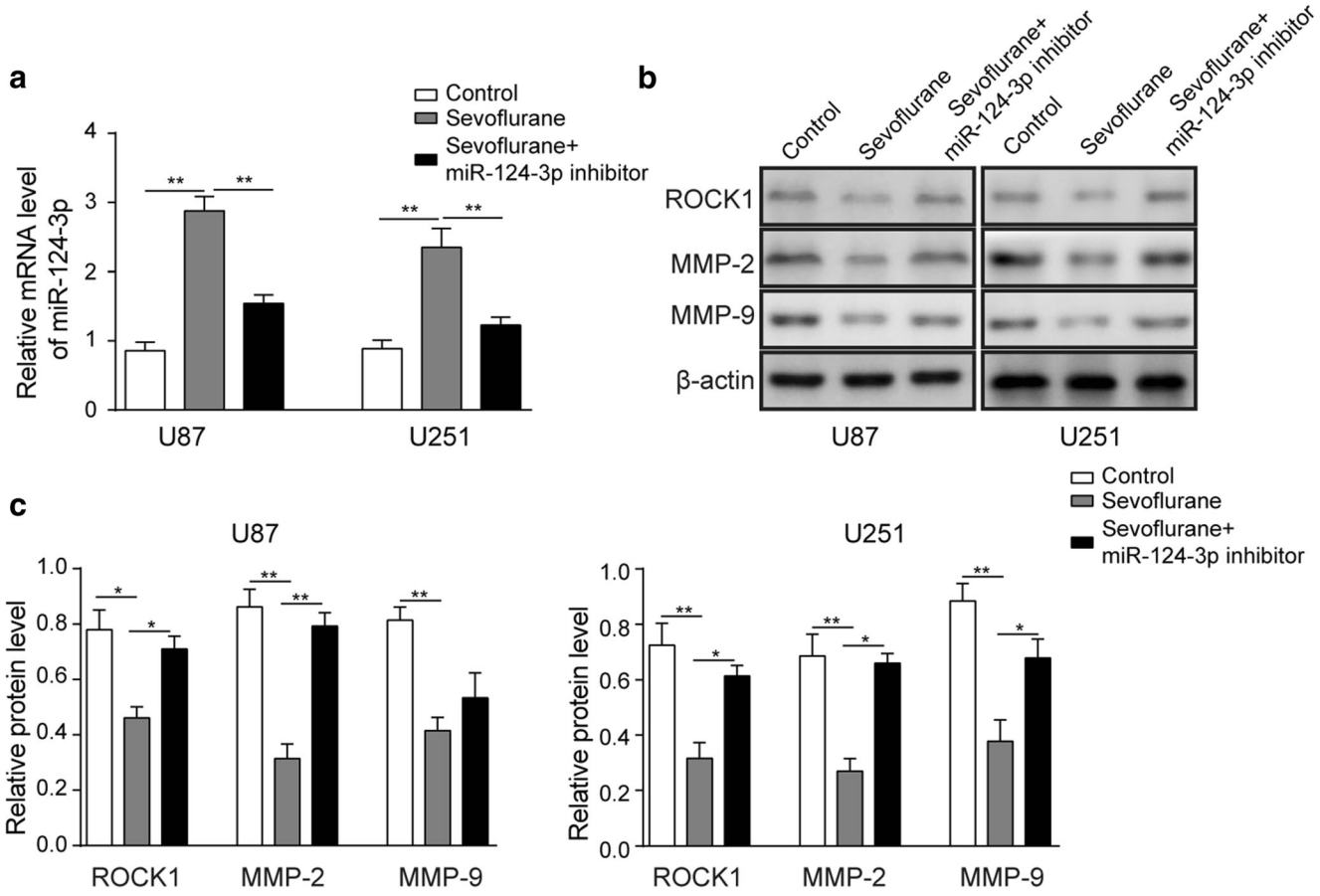

Fig. 4 Sevoflurane suppress the ROCK signaling pathway through up-regulate miR-124-3p. a qRT-PCR analysis of relative miR-124-3p levels in miR-124-3p transfected sevoflurane treated U251 and U87 cells. **b** Western blot analysis of the expression levels of ROCK1 protein and its downstream protein in miR-124-3p transfected U251 and U87 cells treated with sevoflurane. **c** qRT-PCR analysis of ROCK1 and its downstream gene mRNA levels in miR-124-3p transfected sevoflurane treated U251 and U87 cells. $^{**}P < 0.01$, $^{*}P < 0.05$, $n = 3$

the most effective ways to treat gliomas. However, due to limitations in the function and structure of brain tissue and the formation of tumor cell chemo-radiotherapy resistance, the majority of patients are refractory to recurrence [1]. The patient's 5-years survival rate is less than 5%. As an important part of the operation, anesthesia will influence the recurrence and metastasis of malignant tumors and have an impact on the treatment and prognosis of cancer patients [2]. In recent years, anesthesia has gradually become a focus of attention and research of anesthesiologists. Clinical studies have demonstrated that anesthesia and anesthesia techniques affect postoperative recurrence of tumors, but the exact mechanism remains unclear. The purpose of this study was to investigate the effects of sevoflurane on glioma cells, such as the regulation of cell proliferation, invasion and migration.

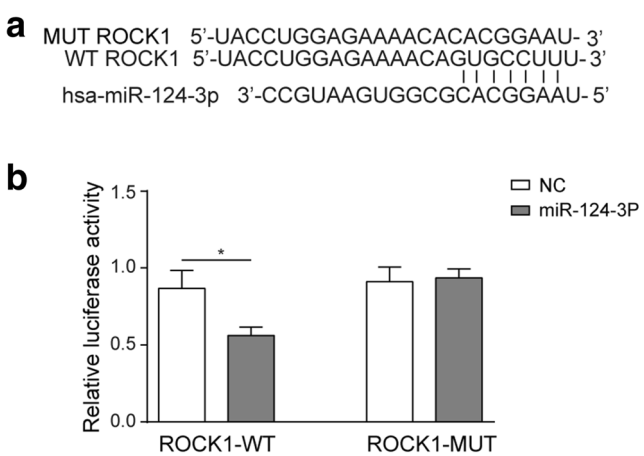

Fig. 5 MiR-124-3p directly target ROCK1 gene. a The predicted binding sites in ROCK1 and miR-124-3p. **b** Luciferase reporter containing either wild-type (WT) or mutant (MUT) ROCK1 was co-transfected into U251 cells with a control mimics and miR-124-3p mimics. $^{**}P < 0.01$, $^{*}P < 0.05$, $n = 3$

Sevoflurane is a widely used inhaled anesthetic in clinical practice. Studies have shown that it can selectively relax cerebral vascular smooth muscle, reduce cerebral vascular resistance, and increase oxygen supply and perfusion of brain tissue during anesthesia [9]. Early studies have shown that sevoflurane can inhibit the proliferation of colon carcinoma cells SW620 and CACO-2 [10]. In colorectal cancer, the volatile anesthetics sevoflurane and desflurane can inhibit the

release of MMP-9 from neutrophils and further attenuate the migration of cancer cells [11]. Liang et al. showed that sevoflurane could inhibit the invasion and metastasis of non-small-cell lung cancer by inhibiting the expression of p38/MAPK, hypoxia-inducible factor (HIF)-1α and MMP family and could increase the sensitivity of tumor cell radiotherapy [12, 13]. Wei et al. found that sevoflurane could induce apoptosis of human non-small cell lung cancer cell A549, which in turn reduced cell viability and increases apoptotic bodies [14]. The results of these experiments show that the proliferation, invasion and migration of glioma cells were significantly down-regulated by sevoflurane.

MicroRNAs are non-coding small RNAs with a length of 20–23 nucleotides in a living body. They can degrade or inhibit mRNA transcription by complementary pairing with a target gene mRNA, thereby regulating gene expression. As an endogenous gene expression regulation factor, miRNA plays a significant regulatory role in the normal physiological and pathological development of animals [4]. Previous studies have shown that sevoflurane can cause a large number of gene expression changes in the hippocampus [15]. Since miRNAs play a significant role in the regulation of gene expression, changes in gene expression induced by sevoflurane may be related to miRNA regulation of gene expression. In our study, we found that miR-124-3p were significantly up-regulated after sevoflurane treated. MiR-124 is abundant in the nervous system and is widely present in the brain, retina, and myelin [16]. In 2006, Wang X et al. used bioinformatics methods and the Northen blot to discover that the miR-124 target genes contained a large number of cell cycle-related genes [17]. The effect of miR-124 on humans was extended to tumors. Silber et al. found that miR-124 and miR-137 were significantly reduced in glioblastoma multiforme and anaplastic astrocytoma and increased by 8–20 times in the differentiation of brain tumor stem cells. The transfection of miR-124 or miR-137 in the glioma cell lines U251 and SF6969 inhibited the G1/S phase transition of cells, accompanied by decreased CDK6 expression and phosphorylation of Rb protein, suggesting that miR-124 and miR-137 may become Potential therapeutic targets for GBM [18]. Pierson et al. found that the expression of miR-124 in the myeloid cell lines was significantly lower than in normal adult cerebellum, and that CDK6 was the target of miR-124 in medulloblastoma cells by the fluorescein reporter system. Expression of miR-124 can reduce the expression of CDK6 and slow down the growth of tumor cells. The decrease of miR-124 expression is also detected in medulloblastoma tissue. This study suggests that miR-124 may inhibit tumor cell growth by regulating CDK6 [19]. Skalsky RL et al. demonstrated that miR-124 could inhibit the growth of glioblastoma and analyzed the differential expression of miR-124 in different glioma cell lines and its molecular mechanism of inhibiting glioma cell growth [20]. Our study indicated that the up-regulation of miR-124 expression is associated with inhibition of sevoflurane on proliferation, invasion and migration of glioma cells. What's more, inhibition of miR-124 could abolish the inhibition of sevoflurane on migration and invasion of glioma cells. These finding indicate that sevoflurane inhibits the proliferation, invasion and migration ability of glioma cells by up-regulating the expression of miR-124.

ROCK1 is a downstream effector molecule of the Rho subfamily and its function involves important aspects such as cell invasion and migration, skeleton rearrangement. In addition, in the process of tumorigenesis, it is also involved in the regulation of cell apoptosis, invasion, and migration, signaling pathways, and plays a variety of cell biological functions [21, 22]. This result demonstrated that sevoflurane down-regulated both the mRNA and protein expression of ROCK1 through enhancing miR-124 level, thereby inhibiting the expression of MMP-2 and MMP-9. In addition, inhibition of miR-124 levels restored sevoflurane-mediated down-regulation of ROCK1, MMP-2 and MMP-9. These findings suggest that sevoflurane controls the ROCK signaling pathway via ROCK1-miR-124-3p axis in glioma cells.

In summary, sevoflurane can inhibit the proliferation, invasion and migration of glioma cells. The mechanism of this action is to up-regulate the expression of miR-124, thereby inhibiting the ROCK1 signaling pathway. The results of the study will help to understand the pharmacological effects of inhaled general anesthetics more comprehensively and help to provide an experimental basis for selecting more reasonable anesthetics for cancer patients.

Funding Guided Science and Technology Project of Changzhou Health and Technology Commission in 2018 (No.: WZ201810).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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