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Fraxinellone inhibits progression of glioblastoma *via* regulating the SIRT3 signaling pathway

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ABSTRACT

Glioblastoma (GBM) is the most prevalent type of adult primary brain tumor and chemotherapy of GBM was limited by drug-resistance. Fraxinellone is a tetrahydro-benzofuranone derivative with various pharmacological activities. However, the pharmacological effects of fraxinellone on GBM remains largely unknown. Here, we found that fraxinellone inhibited the proliferation and growth of GBM cells in a dose-dependent manner *in vitro*. Subsequently, we found that fraxinellone suppressed the migration and induced apoptosis of GBM cells *in vitro*. Using western blot and immunostaining, we further found that fraxinellone downregulated the expressions of sirtuin 3 (SIRT3), and superoxide dismutase 2 (SOD2), a downstream of SIRT3 in GBM cells. Meanwhile, reactive oxygen species (ROS) were increased in these fraxinellone-treated GBM cells. Interestingly, overexpression of SIRT3 (SIRT3-OE) indeed partially restored the inhibition of both cell proliferation and migration of GBM cells induced by fraxinellone. Finally, we found that fraxinellone could inhibit the growth of GBM in xenograft model through the inactivation of SIRT3 signaling pathway. Taken together, these results suggest that fraxinellone suppressed the growth and migration of GBM cells by downregulating SIRT3 signaling *in vitro*, and inhibited the tumorigenesis of GBMs *in vivo*.

1. Introduction

Gliomas are one of the most common types of primary brain tumors [1]. Glioblastoma (GBM) is considered to be a grade IV glioma[2]. Individual glioblastoma cells display an unmatched capacity to invade into surrounding brain areas and thus exert a locally destructive influence on brain tissues and functions[3,4]. These findings, together with the intrinsic and extrinsic heterogeneity of the tumor and its microenvironment, make GBM one of the most difficult cancers to treat. Treatment usually includes surgery, followed by chemotherapy and radiation, though it is hard to cure[5–7]. For this reason, treatment is typically aimed at prolonging life expectancy and making the patients comfortable rather than getting rid of the cancer permanently.

Traditional Chinese medicine can prevent the formation of tumors, reduce tumor recurrence and metastasis, prolong survival time and improve patients' quality of life[8,9]. Its active ingredients and derivatives have made great achievements in the treatment of glioma [10–12], for example, β-elemene, a natural plant drug extracted from Curcuma wenyujin[13], promotes the senescence of glioma cells through regulating YAP-CDK6 signaling[14]. Danshinone can inhibit cell proliferation and enhance its efficacy when used in combination with temozolomide[15–17]. Dictamnus dasycarpus (*D. dasycarpus*) is a traditional Chinese medicine that has been commonly used in the treatment of cancers[18]. Fraxinellone is a natural product isolated from the *D. dasycarpus* plant, which has been shown to exhibit antibacterial and insecticidal activities, and more recently anti-inflammatory and

Abbreviations: FRA, Fraxinellone; GBM, Glioblastoma; SIRT3, Sirtuins3; SIRT3-OE, Overexpression of SIRT3.

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anticancer activities[19]. Recent study has shown that it can induce the downregulation of the TGFβ signal transduction pathway to inactivate cancer-related fibroblasts, and cause a decrease in the number of M2 macrophage[20]. Fraxinellone is a tetrahydro-benzofuranone derivative substituted with methyl and furan groups which lie on the same side of the fused ring system[21]. Several studies have shown that fraxinellone has an anti-tumor effect. It inhibited the proliferation and migration of osteosarcoma cells HOS and MG63 in a dose-dependent manner[22]; and in lung cancer cells, fraxinellone inhibited PD-L1 expression by downregulating the STAT3 and HIF-1α signaling pathways, subsequently inhibiting cell proliferation and angiogenesis[19,23]. However, the roles of fraxinellone in GBM cells remain unclear.

Sirtuin 3 (SIRT3), the major deacetylase in mitochondria, serves an essential role in the maintenance of mitochondrial network integrity by activating the antioxidant machinery and coordinating mitophagy[24]. SIRT3 shows a dual role in cancers, as it can act as a tumor suppressor or a tumor promoter, depending on the cellular context[25]. For example, in breast cancer, some studies suggest that a reduction in SIRT3 levels is required to elevate the levels of reactive oxygen species (ROS) and assist in metabolic reprogramming during malignant transformation while SIRT3 acts to lower ROS levels below a critical threshold and retain cellular viability[26]. Importantly, previous studies have shown that linalool exhibited inhibitory effects on glioma cells through regulation of SIRT3-SOD2-ROS signaling. The mRNA and protein expression of SIRT3 were significantly inhibited by linalool[27]. Furthermore, Ke Luo, et al.[28] have demonstrated that the higher SIRT3 protein levels indicated the poorer overall survival of glioma patients. Meanwhile, SIRT3 enhances glioma cell viability by stabilizing Ku70-BAX interaction. However, it remains unknown whether fraxinellone plays anti-GBM effects through SIRT3 signaling.

In the present study, we explored the antitumor effects of fraxinellone on GBM cells both *in vitro* and *in vivo*. We found that fraxinellone inhibited proliferation and migration, induced apoptosis and senescence of GBM cells through downregulation of SIRT3 signaling. *In vivo* observations were confirmed by showing antitumor activity of fraxinellone in a murine xenograft model. Our findings indicate that fraxinellone can be a valuable candidate for the development of a cancer therapeutic for GBM.

2. Results

2.1. Fraxinellone inhibited the proliferation and growth of GBM cells in vitro

We initially evaluated the effects of fraxinellone on the proliferation and growth of C6 and DBTRG cells (cell lines of GBM) *in vitro*. As shown in Fig. 1A-B, CCK8 assays revealed that fraxinellone significantly decreased C6 and DBTRG cells viability in dose-dependent and time-dependent manners. Furthermore, immunocytochemistry showed that fraxinellone treatment dramatically reduced the percentage of PH3 (a marker of mitotic cells) positive cells of C6 and DBTRG cells in a dose-dependent manner (Fig. 1C-F), suggesting that fraxinellone reduced the proliferation of GBM cells. To further determine the anti-proliferation effects of fraxinellone, a colony formation assay was performed and results showed that fraxinellone inhibited the growth of C6 and DBTRG cells (Fig. 1G, H). Taken together, these results suggested that fraxinellone inhibited the proliferation and growth of GBM cells *in vitro*.

2.2. Fraxinellone arrested cell cycle of GBM cells at G0/G1 phase

To further confirm whether fraxinellone inhibited cell proliferation by inducing cell cycle arrest, flow cytometry was performed. The results showed that fraxinellone treatment induced G0/G1 phase arrest in C6 and DBTRG cells, which was accompanied by a decrease in the number of cells at the G2/M and S phases (Fig. 2A, B). Furthermore, western blot analysis showed that the levels of Cyclin D1 expression were significantly decreased in fraxinellone treated C6 and DBTRG cells (Fig. 2C, D). Taken together, these results suggested that fraxinellone treatment potently induced G0/G1 phase arrest in GBM cells.

2.3. Fraxinellone triggered cell senescence and death in GBM cells

We further examined the effects of fraxinellone on GBM cell senescence by SA-β-Gal staining (a staining test of senescent cells or tissues based on the upregulation of senescence-associated β-galactosidase activity levels in aging) and apoptosis by flow cytometry assays and PI staining. The results showed that fraxinellone treatment significantly increased the percentages of the cell senescence (Fig. S1), the early apoptotic cells and the late apoptotic cells in C6 and DBTRG cells (Fig. 3A, B). PI staining also showed that fraxinellone treatment significantly increased the death of C6 and DBTRG cells (Fig. 3E-G). Furthermore, western blot showed the level of cleaved caspase 3 expression was significantly increased in fraxinellone treated C6 and DBTRG cells (Fig. 3C, D). Immunofluorescence results also showed that the cleaved caspase 3^{+} cells was significantly increased in C6 and DBTRG cells after treated with fraxinellone (Fig. 3H-K). Taken together, these results suggested that fraxinellone treatment triggered cell senescence and death in GBM cells.

2.4. Fraxinellone suppressed the migration of GBM cells

To investigate whether fraxinellone affects cell migration, wound healing assays were performed. The results showed that treatment with fraxinellone significantly reduced wound closure of C6 and DBTRG cells, compared with that in controls (Fig. 4A-D), suggesting fraxinellone inhibited the migration of GBM cells. What's more, as shown in Fig. S2A-D, we further found that fraxinellone significantly decreased the migration of C6 and DBTRG cells in dose-dependent and time-dependent manners. Notably, the cell viability was not affected in both 20 μM and 50 μM in C6 after 24 h treatment as shown in Fig. 1A, suggesting that fraxinellone can indeed decrease the migration of GBM cell but not because of the cell death.

To further confirm the inhibitive effect of fraxinellone on invasive cell migration, a transwell assay was then performed. As shown in Fig. 4E, F, G, fraxinellone significantly suppressed the migration of C6 and DBTRG cells after exposure for 24 h. Taken together, these results suggested that fraxinellone treatment inhibited the migration of GBM cells.

2.5. Fraxinellone inhibited the tumorigenesis of GBM by regulating the SIRT3-SOD2-ROS signaling

SIRT3 is a novel therapeutic target to treat cancers. According to the survival analysis based on two databases, GBM patients with higher expression of SIRT3 mRNA usually had shorter survival probability (Fig. S3A, B). Therefore, to investigate the mechanisms by which fraxinellone inhibited cell growth and induced cell apoptosis in GBM cells, we analyzed the expressions of SIRT3 which have been demonstrated to be involved in cancer cell proliferation and survival [24]. We firstly examined the mRNA expression of *SIRT3* in each set of GBM cells treated with fraxinellone. RT-qPCR results showed that the mRNA level of *SIRT3* was decreased in these fraxinellone-treated GBM cells (Fig. 5A, B). Furthermore, immunofluorescence results also showed that the intensity of SIRT3 was significantly decreased in C6 and DBTRG cells after treated with fraxinellone (Fig. 5C-F). The results from western blot analysis showed that the levels of SIRT3 protein expression in GBM cells were significantly lower in the fraxinellone treatment group than that in the control group (Fig. 5G, H). These results suggested fraxinellone downregulated the expression level of SIRT3 both at mRNA and protein level.

Additionally, SIRT3 is emerging as a pivotal regulator of oxidative stress by deacetylation of substrates involved in both ROS production

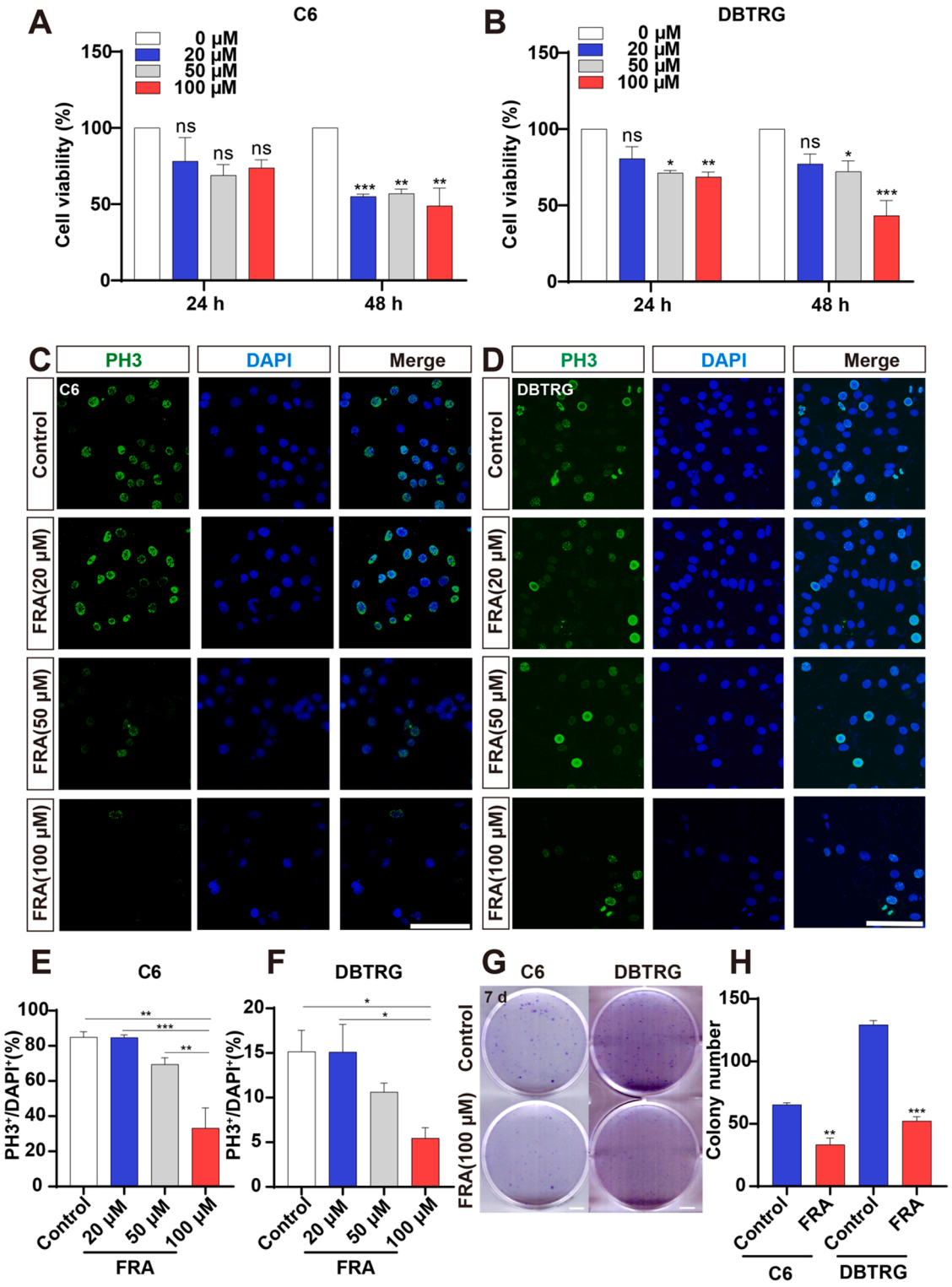

Fig. 1. Fraxinellone inhibited the proliferation and growth of GBM cells *in vitro*. **(A, B)** The cell viability of C6 cells (A) and DBTRG cells (B) treated with different concentrations of fraxinellone detected by CCK8 assays (n = 5/group, two-way ANOVA, Bonferroni's test was performed for the multiple comparison). **(C, D)** Typical images of PH3 staining (green) in C6 and DBTRG glioma cell lines after treated with vehicle control or 100 μM fraxinellone for 48 h. Scale bars, 50 μm. **(E, F)** Quantitative analysis of the percentages of $PH3^+$ cells over total C6 and DBTRG cells in one field as shown in (C) and (D) (n = 5/group, one-way ANOVA, Turkey's test was performed for the multiple comparison). **(G)** Typical images of C6 and DBTRG cells treated with 100 μM fraxinellone for 1 week in the colony formation assays. Scale bars, 500 μm. **(H)** Quantitative analysis of colony number (the surviving colonies >10 cells) as shown in (G) (n = 3/group, *t*-test). Data were mean ± s.e.m. ns, not statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

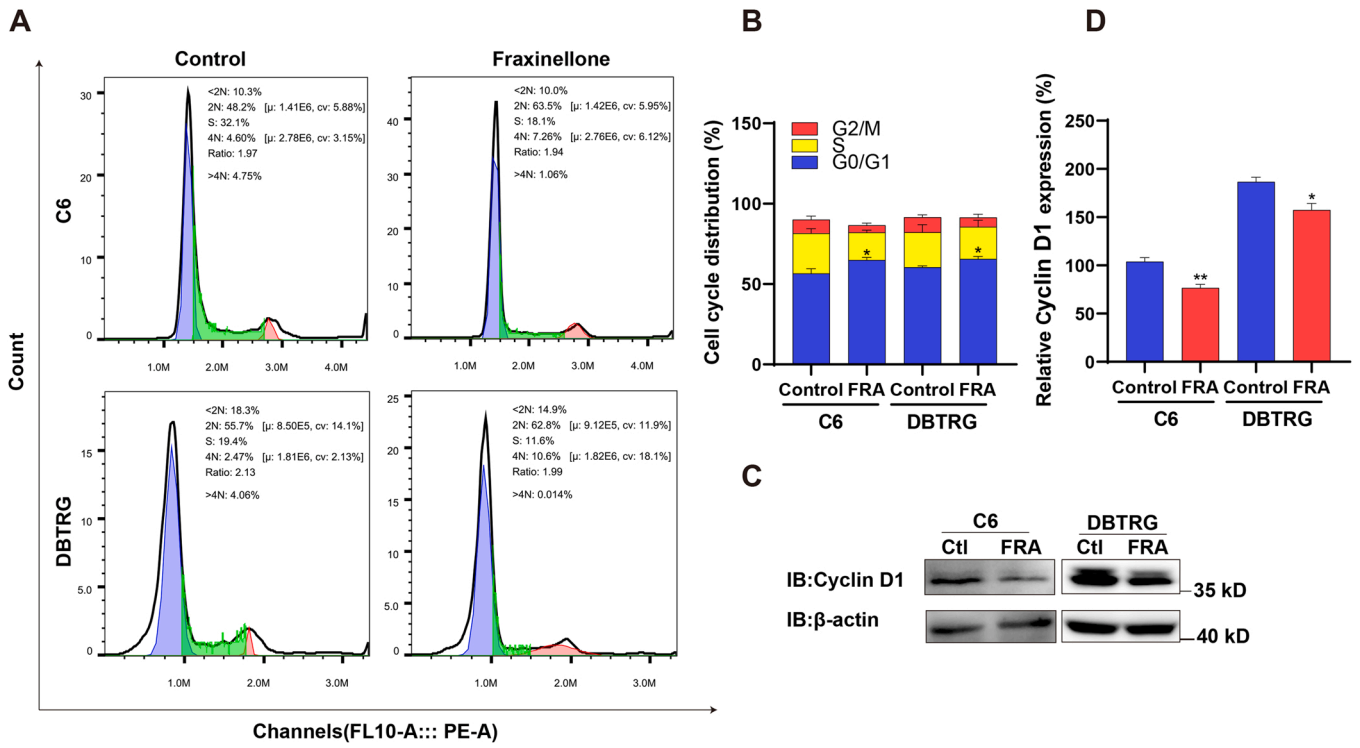

Fig. 2. Fraxinellone arrested cell cycle of GBM cells at G1 phase. **(A, B)** Representative flow cytometry results of cell cycle (A) and quantitative analysis in C6 and DBTRG after fraxinellone treatment for 48 h (B, n = 6/group, *t*-test). **(C)** Western blot detected the expression of Cyclin D1 in C6 and DBTRG cells treated with fraxinellone for 48 h. **(D)** Quantification analysis of the relative Cyclin D1 level in C6 (n = 3/group, *t*-test) and DBTRG (n = 4/group, *t*-test) as shown in (C). Data were mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$.

and detoxification[29]. Previous studies have shown that SIRT3-SOD2-ROS signaling was involved in the regulation of glioma [27]. ROS is a known inducer of the transcription of specific genes involved in cell death[30–32]. SOD2 is an important antioxidant enzyme located in mitochondria and plays a critical role in protecting mitochondria against potential oxidative insult[27]. Therefore, we then analyzed the intracellular ROS and SOD2. The results revealed that the expression of SIRT3 and SOD2 were both reduced in C6 and DBTRG cells after treated with fraxinellone (Fig. 5G-I) and intracellular ROS production was increased in C6 cells (Fig. 5J, K). Taken together, these findings suggested that fraxinellone might inhibit the tumorigenesis of GBMs through downregulation of SIRT3 signaling.

2.6. Fraxinellone inhibited the tumorigenesis of GBM by downregulation of SIRT3 signaling

To further confirm the roles of SIRT3 in the regulation of GBM tumorigenesis by fraxinellone, a plasmid for overexpression of SIRT3 was constructed. As expected, the expression of SIRT3 was significantly upregulated in C6 cells transfected with SIRT3 (Fig. 6A, B). Interestingly, as shown in Fig. 6C, D, E, overexpression of SIRT3 (SIRT3-OE) indeed partially restored the inhibition of both cell proliferation and migration of C6 cells by fraxinellone treatment. Taken together, these results strongly suggested that fraxinellone inhibited the proliferation and migration of GBM by downregulating SIRT3 signaling.

2.7. Fraxinellone inhibited tumor growth in vivo by inactivating SIRT3 signaling

In view of *in vitro* experiments showing significant anti-GBM activity of fraxinellone, we further confirmed this effect of fraxinellone in xenograft GBM models. A subcutaneous glioblastoma tumor mouse model was established, and the tumor-bearing mice were received intragastric administration of fraxinellone at the dose of 50 mg/kg for 23 d. Fraxinellone significantly decreased tumor volume, compared with that of the model group *in vivo* (Fig. 7A, F). Meanwhile, consistent with the results *in vitro*, the expression of Cyclin D1 and SIRT3 was significantly downregulated, and the expression of cleaved caspase-3 was increased in these tumors by fraxinellone treatment, compared with control groups (Fig. 7C-E). Taken together, these results suggested that fraxinellone inhibited the tumorigenesis of GBMs by downregulation of SIRT3 signaling *in vivo*.

To assess the *in vivo* side effects of fraxinellone, various organs were collected. Organs were sectioned and stained with H&E. No histological differences in the lung, heart, liver, kidney, or spleen were found in the fraxinellone treatment groups, indicating that there was no notable toxicity (Fig. S4).

3. Discussion

Our studies revealed that fraxinellone suppressed the growth and migration of GBM cells by downregulating SIRT3 signaling *in vitro,* and inhibited the tumorigenesis of GBMs by downregulating SIRT3 signaling *in vivo*. Our data will hopefully support the use of fraxinellone as a potential compound for GBM treatment.

Dictamnus dasycarpus has been commonly used in China for the treatment of inflammation, microbial infection, pruritus vulvae, eczema, scabies, cancer, and other diseases for thousands of years, in which fraxinellone is one of the main active ingredients[33]. Fraxinellone has been reported to be neuroprotective and have anti-inflammatory functions[34]. Besides, it also has antitumor effects on lung cancer, osteosarcoma and desmoplastic melanoma[19,22,35]. Nevertheless, the pharmacological effect of fraxinellone on GBM has rarely been reported.

Glioma is the most common primary malignant brain tumor in adults and the patients have poor prognosis despite treatment with surgery,

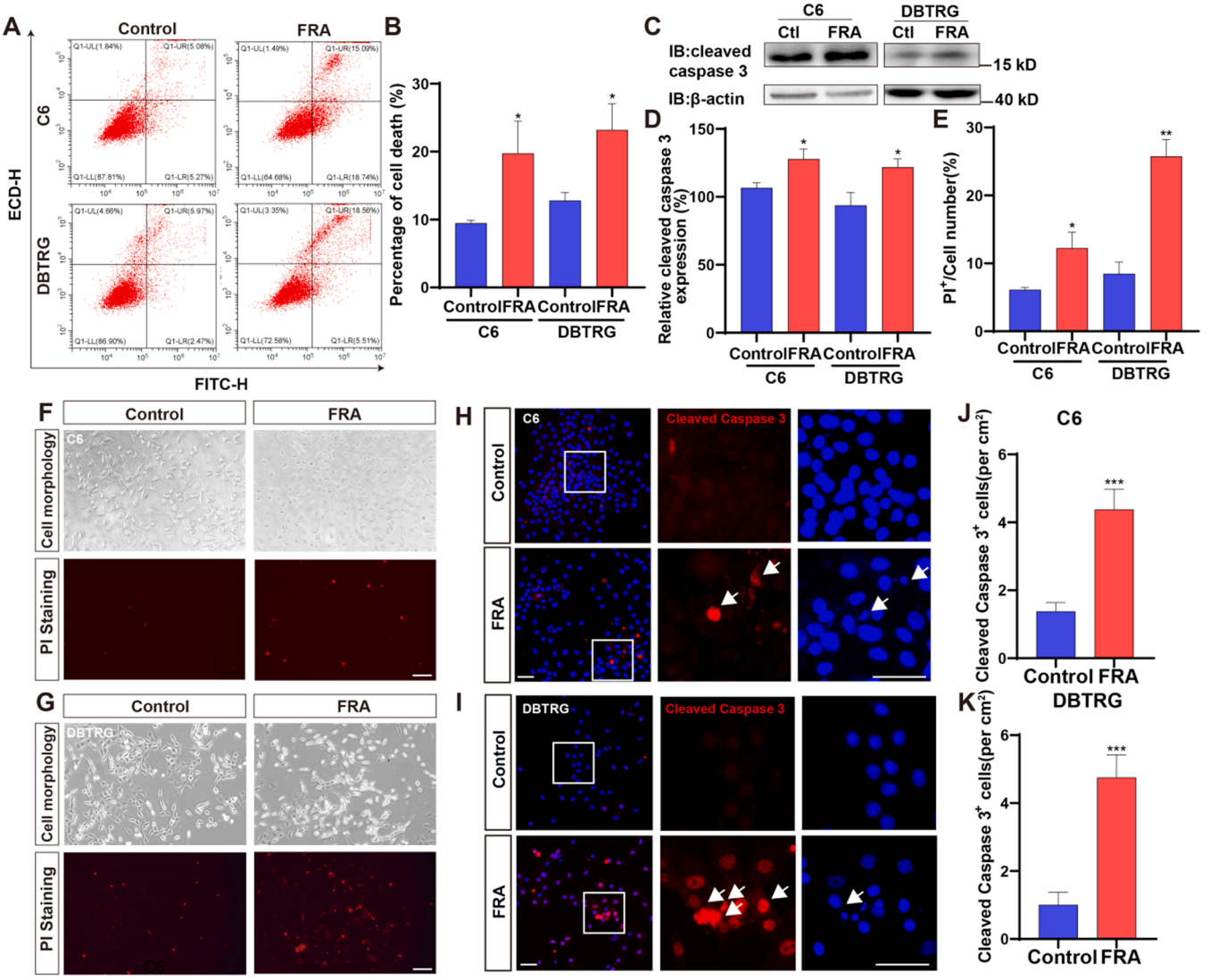

Fig. 3. Fraxinellone triggered cell death in GBM cells. (**A**)Representative results of annexin V/FITC/PI staining in C6 and DBTRG cells treated with fraxinellone for 48 h in flow cytometry assays. **(B)** Quantitative analysis of the percentages of apoptotic cells in C6 (n = 4/group, *t*-test) and DBTRG (n = 6/group, *t*-test) cells. **(C)** Western blot detected the expression of cleaved caspase 3 in C6 and DBTRG cells treated with fraxinellone. **(D)** Quantification analysis of the relative cleaved caspase 3 level in C6 (n = 4/group, *t*-test) and DBTRG (n = 8/group, *t*-test) cells as shown in C. **(E)** Quantitative analysis of percentage of PI^+ cells in one filed as shown in (F) and (G) (n = 4/group, *t*-test). **(F, G)** Representative image of PI staining in C6 (F) and DBTRG (G) cells after treated with vehicle control or 100 μM fraxinellone for 48 h. Scale bars, 200 μm. **(H, I)** Representative images of the immunofluorescence results of cleaved caspase 3 (red) in C6 (H) and DBTRG (I) cells after treated with vehicle control or 100 μM fraxinellone for 48 h. Scale bars, 50 μm. **(J, K)** Quantitative analysis of percentage of cleaved caspase 3^+ cells in one filed as shown in (J) and (K) (n = 8/group, *t*-test). Data were mean ± s.e.m. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

radiotherapy and chemotherapy[36]. It is a serious threat to human health and puts a heavy burden on families and society. Hence, novel therapeutics are required for conditions under which current cancer therapies are inadequate. In recent decades, traditional Chinese medicine and its active ingredients have become important sources for the development of new anticancer drugs[37]. Therefore, finding a small molecule that is easy to produce, orally active and cheaper than biotechnological protein products to effectively treat glioma can not only reduce the cost of cancer treatment but also improve the quality of life of patients. In this context, the case of the natural product fraxinellone is interesting to be considered, as an anticancer agent with immuno-regulatory functions and a capacity to remodel the tumor microenvironment[18,38]. It is a simple, easy to produce and orally active natural product which has recently demonstrated marked anticancer activities *in vivo*. In this study, our results showed that fraxinellone inhibited not only the proliferation and growth of GBM cells *in vitro* and *in vivo*, but also their migration *in vitro*, it may be a candidate compound for treating GBM.

Previous studies[19] showed that the fraxinellone inhibits PD-L1 expression by downregulating the HIF-1α signaling pathway, we have also examined the pathway. However, the results showed that the levels of HIF-1α protein expression in DBTRG cells and xenograft tumors were no significantly change (Figs. S5A-B). We also found no significantly decreased levels of *PD-L1* mRNA after fraxinellone treatment (Figs. S5C), suggesting that fraxinellone plays anti-glioma effects not through the PD-L1/HIF-1α pathway in GBMs.

SIRT3 is an emerging instrumental regulator of the mitochondrial adaptive response to stress, such as metabolic reprogramming and antioxidant defense mechanisms[39]. Metabolic reprogramming, as part of the cancer features, gives cancer cells the potential to grow and proliferate in a nutrient-deficient tumor microenvironment (TME) [40–43]. As mentioned earlier, fraxinellone is an anticancer drug candidate with a capacity to remodel the TME. Therefore, SIRT3 may be a potential target for glioma of fraxinellone treatment. As shown in our

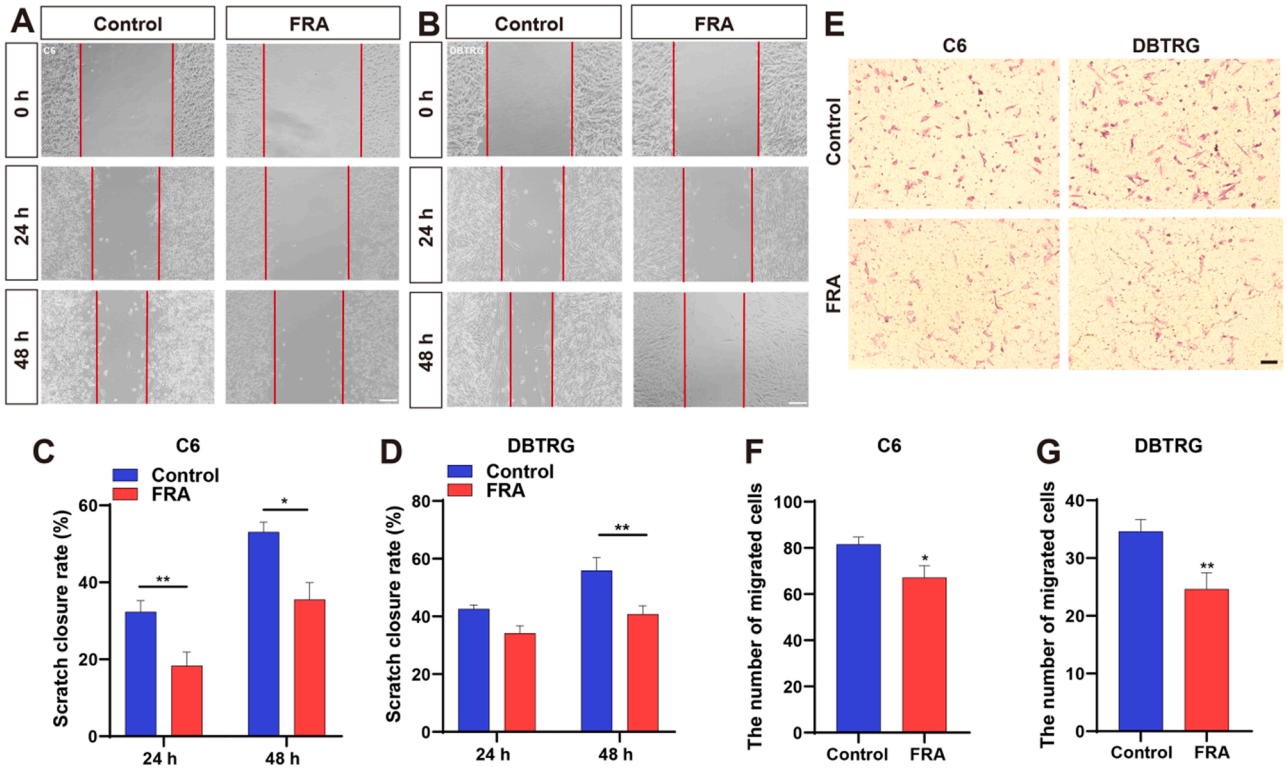

Fig. 4. Fraxinellone suppressed the migration of GBM cells. **(A, B)** Representative images of C6 and DBTRG cells treated with 100 μM fraxinellone in wound healing assays. Phase-contrast images were acquired at 0, 24 and 48 h after scratching and representative images of three independent experiments were shown. Scale bars, 200 μm. **(C, D)** Quantitative analysis of the wound healing area of C6 and DBTRG cells treated with fraxinellone as shown in (A-B) (n = 10/group, two-way ANOVA, Bonferroni's test was performed for the multiple comparison). **(E)** Typical images of C6 and DBTRG cells treated with 100 μM fraxinellone for 24 h in transwell migration assay. Scale bar, 100 μm. **(F, G)** Quantitative analysis of the numbers of migrated C6 (n = 10/group, *t*-test) and DBTRG (n = 15/group, *t*-test) cells counted in representative high-power fields per transwell plate. Data were mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$.

results, fraxinellone increased the expression of cleaved-caspase 3 and reduced the expression of Cyclin D1 and SIRT3, leading to the increase of cell apoptosis. Overexpression of SIRT3 significantly inhibited fraxinellone-induced inhibition of cell migration and decrease of cell viability. In summary, these results demonstrated that fraxinellone exhibited inhibitory effect on glioma cells through regulation of SIRT3. It is well known that SIRT3 is an NAD^{+}-dependent deacetylase linked to a broad range of physiological and pathological processes, including aging and aging-related diseases[44,45]. Consistent with these results, our experimental results also demonstrate that fraxinellone induce the senescence of GBM cells (Fig. S1).

Recently, SIRT3 was reported to play both tumor-suppressing and tumor-promoting roles in different tumor types. Papa et al. [26] demonstrated that in breast cancers, SIRT3 acts to orchestrate two pathways, the antioxidant machinery and mitophagy. A reduction in SIRT3 levels is required to elevate the levels of ROS and assist in metabolic reprogramming during malignant transformation. An increase in SIRT3 levels is required to reduce the levels of ROS below a critical threshold and retain cellular viability. Data support that SIRT3 can function as a tumor promoter or suppressor, depending on the cell- and tumor type and the presence of different stress or cell death stimuli. Also, genetic and environmental factors underlying cancer initiation and development in each patient may contribute to this discrepancy[46,47]. SIRT3 was identified to be expressed higher in glioma tissues than in normal brain tissues, which was significantly correlated to advanced tumor stages. Besides, the higher SIRT3 protein levels indicated the poorer overall survival of glioma patients[28]. Previous research has shown that SIRT3-induced deacetylation of SOD2 and confirmed that downregulation of SIRT3, increase of the acetylation of SOD2 and ROS were involved in linalool induced anti-tumor effects in glioma[27]. In agreement with these observations, we also found that in GBM cells, SIRT3 and SOD2 expression was reduced and ROS were increased after fraxinellone treatment (Fig. 5). However, in this study, we found that the contribution of SIRT3 on the suppressive effect of fraxinellone for GBM cells is partial (Fig. 6), suggesting that SIRT3 is not the only target of fraxinellone. Previous studies have shown that fraxinellone can induce the reversion of cancer associated fibroblasts from an activated stage to the quiescent stage, *via* a drug-induced downregulation the TGFβ signaling pathway. The down-regulation of TGFβ induced by fraxinellone is important because this cytokine not only plays a role in the activation and infiltration of macrophages and their M2 polarization, but also for activation and infiltration of myeloid-derived suppressor cells and regulatory T cells in the tumor microenvironment[21]. Whether TGFβ signaling is also involved in the suppressive effect of fraxinellone needs further exploration.

In the current study, we demonstrated the *in vitro* effectiveness of fraxinellone in two GBM cell lines C6 and DBTRG, and its *in vivo* anti-tumor efficacy in a xenograft mouse model (Fig. 8A). We confirmed that fraxinellone exhibited its efficacy *via* the SIRT3 pathway (Fig. 8B). Our study further reinforces the notion that targeting SIRT3 may be a useful anti-cancer therapeutic approach, and also provides a new chemical entity with pharmacological potential, and provide a potential direction for traditional Chinese medicine in the treatment of GBM.

4. Materials and methods

4.1. Cell culture and animals

The glioma cell lines C6 and DBTRG were kindly provided by Prof. Maojin Yao (Sun Yat-Sen University, Guangdong, China), and were cultured in Dulbecco's Modified Eagle's Medium supplement with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco) at 5% CO_2 at 37 °C.

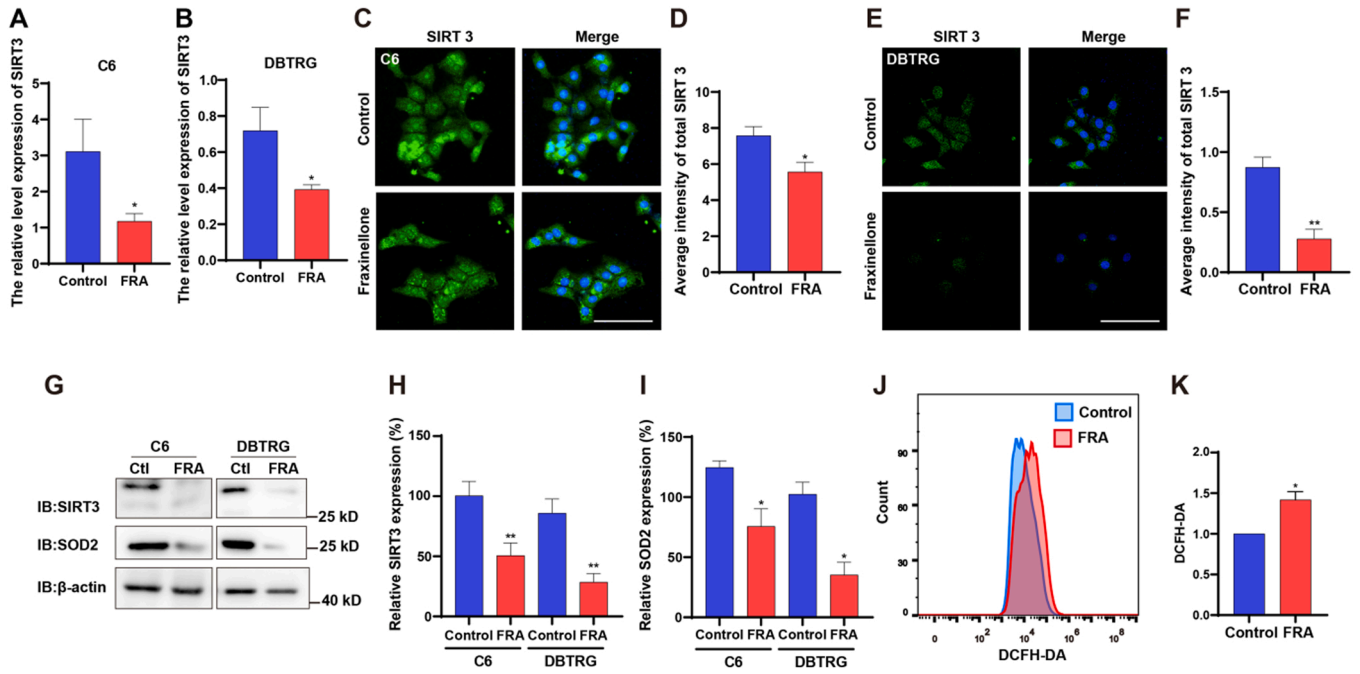

Fig. 5. Fraxinellone inhibited the tumorigenesis of GBM by regulating the SIRT3-SOD2-ROS signaling. **(A, B)** The mRNA expression of SIRT3 in C6 and DBTRG was detected after treated with 100 μM fraxinellone for 48 h by RT-qPCR (n = 6/group, *t*-test). **(C)** Representative images of the immunofluorescence results of SIRT3 (green) in control and C6 cells (treated with 100 μM FRA for 48 h). Scale bar, 100 μm. **(D)** Quantitative analysis of average intensity of total SIRT3 in C6 cells (n = 4/group, *t*-test) as shown in (C). **(E)** Representative images of the immunofluorescence results of SIRT3 (green) in control and DBTRG cells (treated with 100 μM FRA for 48 h). Scale bar, 100 μm **(F)** Quantitative analysis of average intensity of total SIRT3 in DBTRG cells (n = 4/group, *t*-test) as shown in (E). **(G)** The expression of SIRT3 and SOD2 in C6 and DBTRG was detected after treated with 100 μM fraxinellone for 48 h by western blot. **(H, I)** Quantification analysis of relative SIRT3 and SOD2 in C6 and DBTRG cells (n = 3/group, *t*-test) as shown in (G). **(J)** Representative results of ROS analysis in C6 cell line after 100 μM fraxinellone treatment. **(K)** Quantitative analysis of ROS in C6 (n = 3/group, *t*-test) GBM cell line as shown in (J). Data were mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$.

BALB/C-nude male mice (18 ~ 22 g, 6 ~ 8 weeks) were purchased from Shanghai SLAC Laboratory Animal Co.,Ltd, (Shanghai, China). The mice were housed in a standard laboratory condition (temperature 22 ± 2 °C and humidity 50 ~ 60%) at the laboratory animal center of Hangzhou Normal University.

4.2. Drugs and plasmid

Fraxinellone was purchased from MedChemExpress (#HY-N0242). A stock solution of 500 mM was prepared in Dimethyl sulfoxide and stored at − 80 °C until use. The SIRT3 OE plasmid (pip-2xFlag-SIRT3) were purchased from BRICS (http://www.brics.ac.cn/plasmid/template/plasmid/plasmid_detail.html?id=41814). We used it for overexpression and rescue experiments.

4.3. Cell viability assay

The cell viability was detected by CCK-8 cell counting kit (A311–01/02, Vazyme Biotech, Nanjing, China). Briefly, cells were seeded into 96-well plates at a density of approximately 3000 cells per well and cultured for 24 h, then were starved for 24 h with serum free media, and were added different concentrations of fraxinellone followed by culturing for another 24 h. Subsequently, 10 μL CCK-8 solution was added to each well and incubated at 37 °C for 2 h. The optical density of cells was measured at a wavelength of 450 nm using a microplate reader (Varioskan Flash, Thermo scientific, USA).

4.4. Cell cycle assay

Cell cycle was measured by using the Cell Cycle Staining Kit (#A01031, MULTI SCIENCES, Hangzhou, China) following the manufacturer's instructions after fraxinellone treatment. Briefly, cells were re-suspended and mixed in DNA staining solution. After incubation for 30 min, cell cycle was detected with a flow cytometry (CytoFLEX S, Beckman Coulter). The results were presented as percentage of cells at each phase of cell cycle.

4.5. Apoptosis assay

Cell apoptosis was measured by using the Annexin V-FITC/PI Apoptosis Detection Kit (#A00947, MULTI SCIENCES, Hangzhou, China) following the manufacturer's instructions after drug treatment. Briefly, cells were re-suspended and mixed in 500 μL PI. After incubation for 15 min, cell apoptosis was detected with a flow cytometry (CytoFLEX S, Beckman Coulter).

4.6. Colony formation analysis

The C6 and DBTRG cell suspensions were planted in a 6-well culture plate at 5×10^2 cells/well respectively and grown for 24 h. The supernatants were removed and the fresh media containing fraxinellone were added. The cells were cultured for 7 d at 5% CO_2 at 37 °C. In the end, the supernatants were removed and the cells were washed with PBS twice. Then the colonies were fixed with 4% paraformaldehyde (PFA) for 15 min. The colonies were stained with crystal violet solution (0.1%) for 30 min. After fixation and staining, the colonies were imaged and counted.

4.7. Transwell and wound-healing assays

For the transwell migration assay, 1×10^5/mL cells were suspended in the upper chamber of a 24-well transwell plate. After 24 h of coculture, the cells were fixed, stained, and counted under a Nikon light microscope (Model Eclipse Ci-S; Nikon Corporation, Tokyo, Japan). For

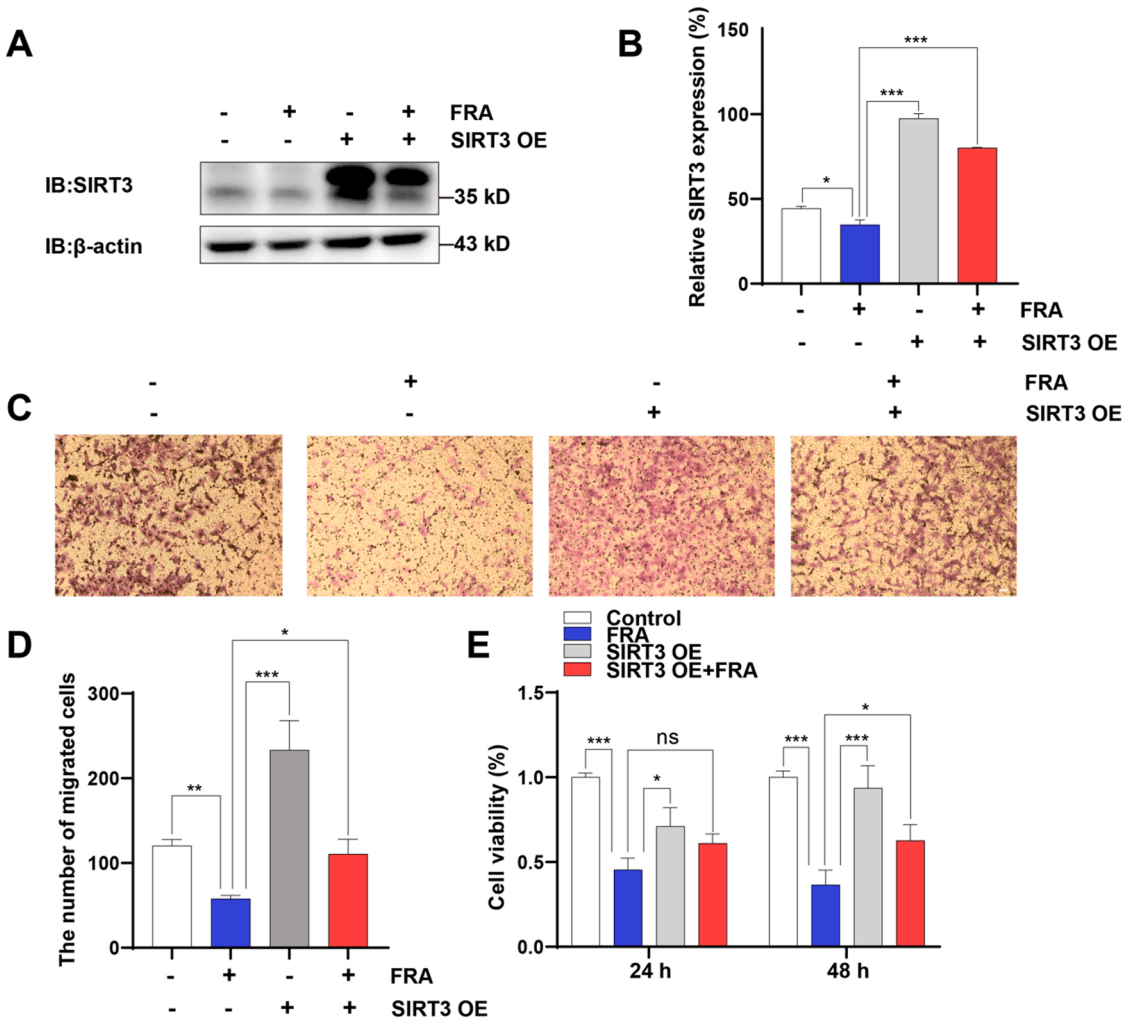

Fig. 6. Fraxinellone inhibited the tumorigenesis of GBM by downregulation of SIRT3 signaling. **(A)** Western blot detected the expression of SIRT3 in C6 transfected with SIRT3 constructs and then treated with 100 μM FRA for 48 h. **(B)** Quantification analysis of the relative SIRT3 level in C6 cells as shown in (A) (n = 3/group, one-way ANOVA, Turkey's test was performed for the multiple comparison). **(C)** Typical images of C6 cells transfected with SIRT3 constructs and then treated with 100 μM FRA for 24 h in transwell migration assay. Scale bar, 50 μm. **(D)** The numbers of migrated cells were counted in representative high-power fields per transwell plate as shown in (C) (n = 9/group, one-way ANOVA, Turkey's test was performed for the multiple comparison). **(E)** The effects of SIRT3-OE on C6 cell viability as detected by CCK8 assays after 24 h and 48 h of 100 μM FRA treatment (n = 4/group, one-way ANOVA, Turkey's test was performed for the multiple comparison). Data were mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

the wound-healing assay, a monolayer of cells at 95% confluence was scratched with a sterile plastic tip and then cultured in serum-free medium.

4.8. Western blotting

Cultured cells were lysed by ice-cold RIPA Buffer (R0010, Solarbio, Beijing, China) and incubated at 4 °C for 30 min. Following centrifugation at 1, 4000g for 20 min, proteins were extracted with 5 × loading buffer and boiled at 100 °C for 15 min. The protein samples then were separated using 10–12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred onto PVDF membranes. After blocking in TBST containing 5% skim milk for 1 h at RT, the immunoblots were incubated with different primary antibodies at 4 °C overnight. Primary antibodies used included rabbit anti-cleaved caspase-3 (#9661, CST, 1:1000), rabbit anti-SIRT3 (#10099–1-AP, Proteintech, 1: 1000), rabbit anti-SOD2 (#ET1701–54, HUABIO, 1: 1000), rabbit anti-HIF-1α (#20960–1-AP, Proteintech, 1: 300), rabbit anti-Cyclin D1 (#55506, CST, 1:1000) and mouse anti-β-actin (#200068–8F10, ZEN BIO, 1:5000) as a loading control was detected alongside the experimental samples. Subsequently, the membranes were washed three times in TBST, and incubated with the horseradish peroxidase (HRP) conjugated secondary antibodies [1:10000, Sigma, A0545 (goat anti-rabbit), A9044 (goat anti-mouse)] for 1 h at RT. After washing in TBST for another three times, the protein signals were detected using the ECL detection kit (#FD8020, FDbio, Hangzhou, China). Blots were analyzed using ImageJ software.

4.9. Immunofluorescence

Cultured cells were rinsed once with PBS, fixed in 4% PFA for 20 min. Then, cells were blocked and permeabilized with 0.1% Triton X-100 in PBS containing 5% normal goat serum (NGS) at room temperature for 1 h. Subsequently, cells were incubated with primary antibodies at 4 °C overnight, washed three times in PBS and then with secondary antibodies at room temperature for 1 h. The primary antibodies included rabbit anti-SIRT3 (#10099–1-AP, Proteintech, 1: 500), mouse anti-PH3 (ab14955, Abcam, 1:2500), rabbit anti-cleaved caspase-3 (#9661, CST, 1:400). After washing in PBS for another three times, cells were mounted. Images were acquired by using a fluorescence microscopy (Olympus VS200).

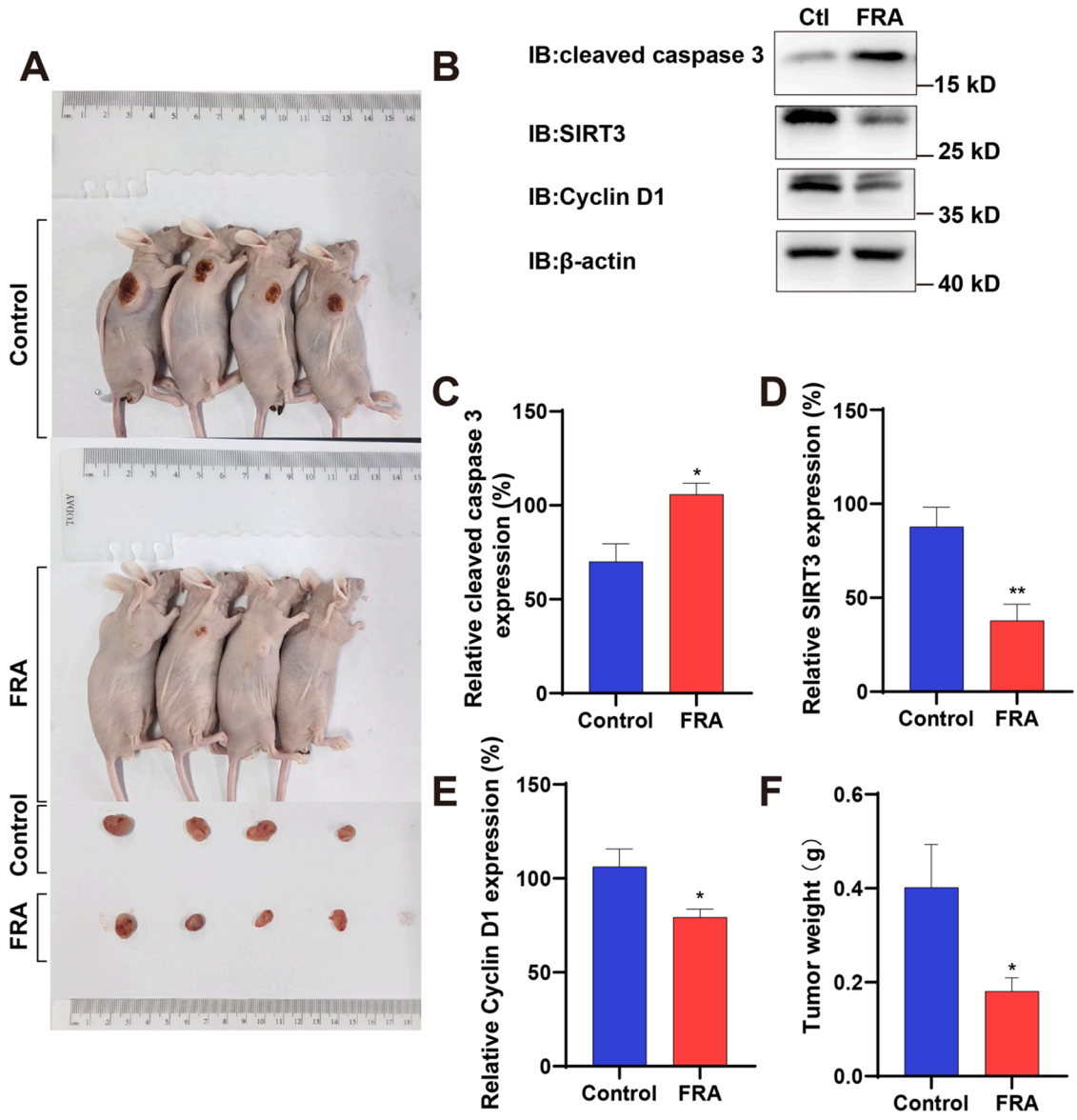

Fig. 7. Fraxinellone exerted its antitumor effects by inactivating SIRT3 signaling *in vivo*. **(A)** Representative images of C6 xenograft tumors after 50 mg/kg fraxinellone treatment for 23 days. **(B)** The expression of cleaved caspase 3, SIRT3 and Cyclin D1 in xenograft tumor tissue was detected by western blot. **(C-E)** Quantification analysis of relative cleaved caspase-3 (C), SIRT3 (D),and Cyclin D1 (E) levels as shown in (B) (n = 5/group, *t*-test). **(F)** Tumor weight in each group (n = 6). Data were mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$.

4.10. *In vivo glioma model and administration of fraxinellone*

A total of 3×10^6 C6 cells were suspended in serum-free DMEM medium and inoculated subcutaneously into nude mice. When the visible tumors at the injection sites grown up to $50 - 100\ mm^3$, the mice were randomly divided into two groups: the vehicle control group and the fraxinellone group. The fraxinellone was dissolved in corn oil and given to the mice by intragastric administration at 50 mg/kg, which dosed at 0.1 mL/10 g of body weight for 23 d. The equal corn oil was given as a control. In the end, the mice were sacrificed by cervical dislocation, the tumors, hearts, liver, lung, and kidney were excised. The tumors were photographed and weighed immediately. Then the tumors and organs were fixed in 4% PFA and subjected to H&E analysis.

4.11. *Senescence-associated β-galactosidase staining*

β-galactosidase staining kit (G1580, Solarbio, Beijing, China) was used to evaluate the senescence of C6 or DBTRG cells. Summarily, the following steps were performed: Cells were first washed twice with PBS and fixed with β-galactosidase fixative solution at room temperature for 15 min. The cells were then washed 3 times with PBS, for 5 min each time, followed by addition of 1 mL dyeing liquid (10 μL β-galactosidase staining fluid A, 10 μL fluid B, 930 μL fluid C, and 50 μL X-Gal solution), and incubated at 37 °C overnight. The cells were finally observed under an inverted microscope (CKX3-SLP, Olympus, Tokyo, Japan). Fine blue uniform particles in the cytoplasm considered β-galactosidase positive signals.

4.12. *Measurement of ROS*

For analysis of intracellular ROS, the redox-sensitive fluorescent probe dichloro-dihydro-fluorescein diacetate (DCFH-DA) was used according to reactive oxygen species assay kit (C3890, APExBIO, Shanghai, China). Cells were incubated with DCFH-DA (1:5000) for 30 min at 37 ℃. The harvested cells were immediately analyzed using a flow cytometry (CytoFLEX S, Beckman Coulter).

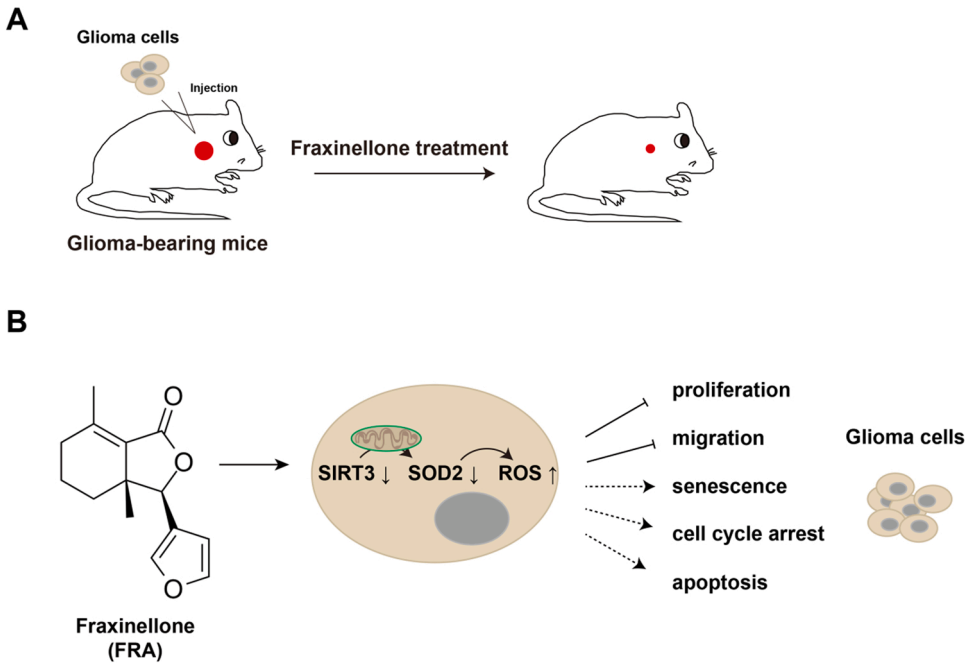

Fig. 8. A working model of the role and mechanism of fraxinellone in anti-GBM. **(A)** Fraxinellone suppresses the growth of GBM cells *in vivo*. **(B)** Fraxinellone inhibits SIRT3 and SOD2 expression and increases intracellular ROS production, and subsequently inhibits the proliferation and migration of GBM cells and probably induces the cycle arrest, apoptosis and senescence of GBM cells.

4.13. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted by RNA-Quick Purification Kit (YISHAN Biotechnology, ES-RN001) according to the manufacturer's protocol. RNA content was quantified by Thermo Scientific™ NanoDrop™ One. cDNA synthesis was performed with the cDNA DyNAmo Kit (R211–01/02, Vazyme, Nanjing, China). qPCR was performed with the SYBR Green PCR master mix (Q511–02/03, Vazyme, Nanjing, China). PCR real time cycling conditions were programmed as such: first step (95 °C, 5 min), cycling step (denaturation 94 °C, 15 s; annealing at 55 °C or 53 °C, 30 s; and final extension for 72 °C, 30 s × 40 cycles), followed by a melting curve analysis to confirm specificity of the PCR. The CT value was corrected by the corresponding β-actin and GAPDH control CT values. The primers used in this study were synthesized by Zhejiang Sunya and presented as follows: PD-L1, 5'-ATGAGGATATTTGCTGTC-3' and 5'-TGTTTTTCTACTGGGAAT-3'; β-actin, 5'-AGACTTCGAGCAGGAGATGGC-3' and 5'-TCGTTGCCAATAGTGATGACCTG-3'; SIRT3 5'-GGCTCTATACACAGAACATCGAC-3' and 5'-TAGCTGTTACAAAGGTCCCGT-3'; GAPDH 5'-AGGTCGGTGTGAACGGATTTG-3' and 5'-GGGGTCGTTGATGGCAACA-3'.

4.14. Statistical analysis

GraphPad Prism 8.0 was used to plot and fit the data. Statistical comparisons were made using Student's *t* test, one-way ANOVA or two-way RM ANOVA (Tukey's test, Bonferroni's test, or Sidak's test was used for *post hoc* comparison) or the Kruskal-Wallis test (Dunn's Multiple Comparison Test was used for *post hoc* comparison). All data are presented as the mean ± s.e.m. In all cases, $p < 0.05$ was considered statistically significant.

Credit author statement

Jiali Shi: Conceptualization, Methodology, Writing - original draft, Visualization, Investigation. **Shanshan Sun:** Conceptualization, Validation, Software, Formal analysis. **Shuqiao Xing:** Resources, Data curation, Software. **Changgang Huang:** Resources, Data curation. **Yuqian Huang:** Resources, Data curation. **Qian Wang:** Resources, Data curation. **Xiumin Xue:** Resources, Data curation. **Zhichao Chen:** Resources, Data curation. **Yongjie Wang:** Conceptualization, Project administration, Funding acquisition, Writing - review & editing. **Zhihui Huang:** Supervision, Project administration, Funding acquisition, Writing - review & editing.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113416.

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