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ORIGINAL ARTICLE

MiR-184 Retarded the Proliferation, Invasiveness and Migration of Glioblastoma Cells by Repressing Stanniocalcin-2

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Abstract To investigate the repression of miR-184 on Stanniocalcin-2 (STC2) and how this axis affects the propagation, invasiveness and migration ability of glioblastoma cells. RT-PCR was employed to determine the miR-184 and STC2 mRNA expression both in tissues and cells. Western blot was employed to determine the protein expression levels. The cells were transfected via lipofection. MTT, colony formation, invasion and scratch healing assays were conducted to study the propagation, invasiveness and migratory ability of glioblastoma cells, respectively. The dual luciferase reporter gene assay was conducted to determine whether miR-184 could directly bind to STC2 mRNA 3'UTR. MiR-184 was under-expressed whereas STC2 was over-expressed in glioblastoma tissues and cell line. The up-regulation of miR-184 significantly suppressed the propagation, migratory ability and invasion of glioblastoma cells, whereas the over-expression of STC2 restored this effect. MiR-184 was confirmed to directly target STC2. MiR-184 could retard the propagation, invasiveness and migratory ability of glioblastoma cells by suppressing STC2.

Keywords Glioblastoma · miR-184 · STC2 · Proliferation · Migration · Invasion

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Introduction

Glioblastoma (GBM), emanating from glial cells or their precursor cells in the central nervous system (CNS), is a very common and malignant form of glioma among adults [1]. GBM accounts for 17% of all types of intracranial tumors and 55% of astrocytic tumors with an estimated 12,120 new cases in 2016 [2]. The median survival time of GBM patients is approximately one year, and the 5-year survival rate is approximately 10% [3], suggesting that GBM is a life-threatening disease. The common clinical manifestation caused by GBM includes headache, nausea, vomiting and other CNS-related complications [4]. In spite of improvements in treatment strategies and processes, they are hard and torturous on GBM patients due to the diffused nature of brain tumors [5]. Therefore, efficient treatment for GBM patients that clarifies the accurate molecular mechanism of pathogenesis is urgent and crucial for the improvement of anti-glioblastoma therapies.

MiRNAs are small endogenous non-coding RNAs comprised of approximately 18–25 nucleotides [6]. MiRNAs can behave as either oncogenes or tumor suppressors [7]. MiRNAs could also affect cell proliferation and apoptosis [8, 9]. Many studies have demonstrated that miR-184 is associated with cell proliferation, invasion and migration in various cancers such as lung cancer, renal cell car carcinoma, epithelial ovarian cancer and human glioma [10–13]. MiR-184 is found down-regulated in A172 and T98G glioma cells, and exogenous miR-184 significantly decreased cell proliferation and invasion [14]. However, the exact mechanism involving miR-184 in glioblastoma remains unclear.

Stanniocalcins (STCs) are secreted glycoprotein hormones that were first found in the corpuscles of Stannius in bony fish [15, 16]. They were reported to be functional in the physiology of Ca^{2+} and phosphate homeostasis, metabolism, stress

response and development [17–19]. In addition, the expression of STC2 has been proved to be involved in a variety of cancers including cervical cancer [20], lung cancer [21], breast cancer [22], hepatocellular carcinoma [23] and nasopharyngeal carcinomas [24].A recent study has demonstrated that STC1, as a new discovered biomarker of glioma progression, is significantly associated with prognosis of patients [25]. Therefore, since STC2 is a mammalian homolog of STC1 and they have been reported to have similar functions in previous studies [26–29], STC2 might possibly have the similar effects in glioma pathogenesis.

In this study, we aimed to investigate how miR-184 affects GBM cells in proliferation, invasion and migration by regulating STC2. The results could possibly contribute to the development of comprehension of GBM pathogenesis and novel GBM treatments.

Methods and Materials

Tissue Samples and Cell Culture

Forty-seven patients with glioblastoma and 30 patients with brain damage were enrolled in this study. They were all accepted for tumor resection surgery during JAN 2014 and JAN 2016 in First Affiliated Hospital of Nanjing Medical University.

Human glioblastoma cell line U87-MG was purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium (DMEM) media at 37°C, 5% CO_2.

RT-PCR

MiR-184 and STC2 mRNA expression analyses were conducted using the miR real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) detection kit (ThermoFisher, MA, USA) and the 7500 Real-Time PCR system. Complementary DNAs (CDNAs) were synthesized by GenePharma company (Shanghai, China). U6 and GAPDH served as the internal controls for miRNA and mRNA, respectively. The relative expression of miR-184 and STC2 were calculated using $2^{-\Delta\Delta C_t}$ method. The primer sequences used in RT-PCR were presented in Table 1.

Cell Transfection and Grouping

Cells were divided into three groups: control-1 group, miR-184 mimics group and miR-NC group. The control-1 cells were transfected with nothing, those in the miR-184 mimics group were transfected with miR-184 mimics, and those in the miR-NC group were lipo-transfected with non-sense sequences. The transfection was performed following the lipofectamine 2000 introduction.

To over-express STC2, pCMV-3tag-3a plasmids were used to construct STC2-flag vectors. The cells were lipo-transfected with pCMV-STC2 vectors (pCMV-STC2 group) or pCMV-3tag-3a plasmids (pCMV-NC group). Another experimental group is the mimics + STC2 group, cells in which were transfected with miR-184 mimics and pCMV-STC2. In addition, the control-2 cells were transfected with nothing.

3-(4, 5-Dimethyl-2-Thiazolyl)-2, 5-Diphenyltetrazolium Bromide (MTT) Assay

Equal numbers of cells (1·10 [5]/well) were plated in 96-well plates. The media were removed carefully after the transfection for 48 h. 200 μl of MTT (5 mg/ml/well) were added and incubated for 6 h at 5% CO_2. Then dimethylsulfoxide (DMSO) (solubilizing reagent, 1 ml/well) was added and incubated for 1 min. The cell viability is visualized by purple color. The suspension was transferred to a spectrophotometer system. Thereafter the optical density (OD) of cells was measured at 570 nm.

Colony Formation Assay

Cells in different groups were plated on 6-well plates at a density of 200 cells/well. Cells were cultured for two weeks to form colonies. At each indicated time point, glioblastoma cells were washed twice in Phosphate Buffered Saline (PBS) and stained with Giemsa. Then the cell culture was photographed and the amount of cell clusters was counted under a microscope (Olympus, Tokyo, Japan).

Transwell Assay

Eight μm-pore inserts (Corning, NY, USA) coated with Matrigel basement were used in the Transwell assay. Fetal bovine serum (FBS) was supplemented to the lower

Table 1 Primer sequences used in RT-PCR

	Forward primer	Reverse primer
MiR-184	5′-TGGACGGAGAACTGATAAGGGT-3'	5′-CCTTATCAGTTCTCCGTCCATT-3'
STC2	5′-TGAAATGTAAGGCCCACGCT-3′	5′-CGAGGTGCAGAAGCTCAAGA-3′
T6	5′ -CTCGCTTCGGCAGCACA-3	5′-AACGCTTCACGAATTTGCGT-3'
GAPDH	5′-ACAACTTTGGTATCGTGGAAGG-3'	5′-GCCATCACGCCACAGTTTC-3'

chambers as stimuli. 5×10^4 cells were allowed to invade for 12 h prior to the fixation. The cells on the upper side of the membrane were removed, whereas those on the down side were stained using crystal violet staining. The number of invaded cells was recorded.

Wound Healing Assay

The cells were grown in 12-well plates until the cell growth reached 90% confluence. The confluent monolayers of cells were scratched using 50 μl pipette tips. The distance between migrating edges was used to quantitate cell migration. Three different locations were visualized and photographed with a phase-contrast inverted microscope (×10, Leica, Germany).

Dual Luciferase Reporter Gene Assay

The wild-type or mutated STC2 3'UTR reporter plasmid pGL3 was co-transfected with negative control miR-184 or miR-184 mimics into U87-MG cells. The cells were treated with Dual-Glo luciferase assay kits (Promega) and the relative luciferase intensities were determined using a LuminoSkan Ascent luminometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) in triplicate. Luciferase activities were expressed as the ratio of firefly luciferase intensity to Renilla luciferase intensity.

Western Blot

Proteins were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to Polyvinylidene fluoride (PVDF) membranes. Primary antibodies (Mouse Stanniocalcin 2/STC-2 Antibody, Clone #348310) were incubated with membranes overnight. Peroxidase conjugated secondary antibodies were then incubated with the membranes for 60 min.

Statistical Analysis

SPSS was used to conduct the data analyses. The continuous data were presented as mean±s.d. Comparison between any two groups were done using Student's t test. The survival rate was analyzed using R. $P < 0.05$ was regarded as significantly different.

Results

The Expression of miR-184 and STC2 in GBM Tissues and Cells

MiR-184 was under-expressed in both glioblastoma tissues and U87-MG cells (Fig. 1a–b), whereas STC2 was over-expressed than in the normal tissues and U87-MG cells (Fig. 1c–d).

MiR-184 Inhibited the Propagation, Invasiveness and Migration of U87-MG GBM Cells

Generally, there was no substantial difference between the control-1 group and the miR-NC group of the relative expression of miR-184, propagation, migrated cell number and invaded cell number.

The expression of miR-184 in mimics-transfected U87-MG cells was dramatically higher than that in U87-MG cells transfected with negative controls (Fig. 2a). The OD at 570 nm of cells transfected with mimics showed substantial suppressed viability than the NC group did (Fig. 2b).

Similarly, the number of colonies and the invasive U87-MG cells per field in the miR-mimics group were dramatically smaller than that in the miR-NC group (Fig. 2c–d).

Besides, the wound closure of the U87-MG cell monolayers demonstrated significantly lower degree than the miR-NC group (Fig. 2e).

These results indicated that miR-184 could prohibit the viability, proliferation, invasion and migration of U87-MG cells.

MiR-184 Directly Targeted STC2 mRNA 3'UTR in U87-MG Cells

Figure 3a illustrated the predicted complimentary sequence of STC2 3'UTR to miR-184, together with the mutated sequence of STC2 3'UTR. The dual luciferase reporter gene assay was conducted to confirm the direct binding relationship between miR-184 and STC2. The relative luciferase intensity in U87-MG cells co-transfected with wild-type STC2 3'UTR and miR-mimics was significantly weaker than that in cells co-transfected with wild-type STC2 3'UTR and miR-NC or cells co-transfected with mutated STC2 3'UTR and miR-mimics (Fig. 3b). RT-PCR and western blot were also conducted to confirm the inhibitory effect of miR-184 on STC2. The relative STC2 expression (both at mRNA level and protein level) was significantly suppressed in U87-MG cells transfected with miR-mimics than those transfected with controls (Fig. 3c).

MiR-184 Retarded U87-MG GBM Cell Propagation, Migration and Invasiveness by Suppressing STC2

RT-PCR and western blot were used to determine the expression of miR-184 and STC2. It was demonstrated that the interference of exogenous STC2 did not affect the expression of miR-184 (Fig. 4a). Besides, cells transfected with pCMV-STC2 showed significant higher STC2 expression level (both at mRNA and protein levels), whereas those transfected with pCMV-STC2 and miR-184 mimics showed lower STC2 expression level than those transfected with pCMV-STC2 (Fig. 4b).

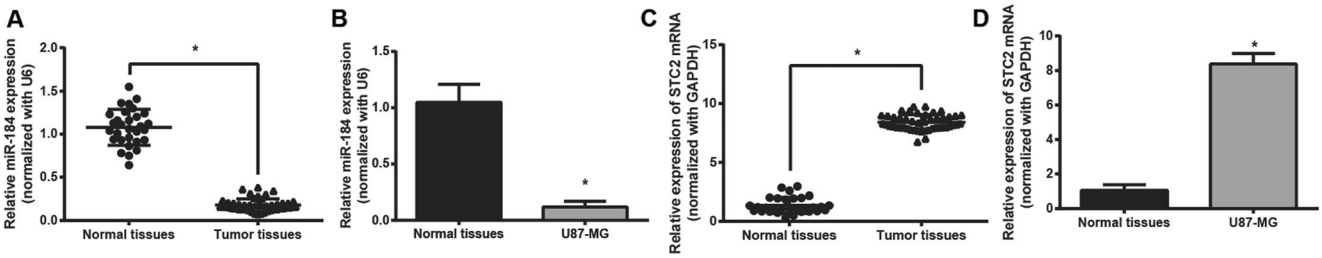

Fig. 1 Expression levels of miR-184 and STC2. **a** RT-PCR results showing the expression of miR-184 in GBM tissues and normal brain tissues. **b** The miR-184 expression in U87-MG cell line was lower than in normal brain tissues. **c** The STC2 mRNA expression in cancerous tissues and normal brain tissues were analyzed by RT-PCR. **d** The STC2 mRNA expression level in U87-MG cell line was higher than in normal brain tissues. U6 served as the internal control for miR-184 and GAPDH served as the internal control for mRNA. ($P < 0.05$)

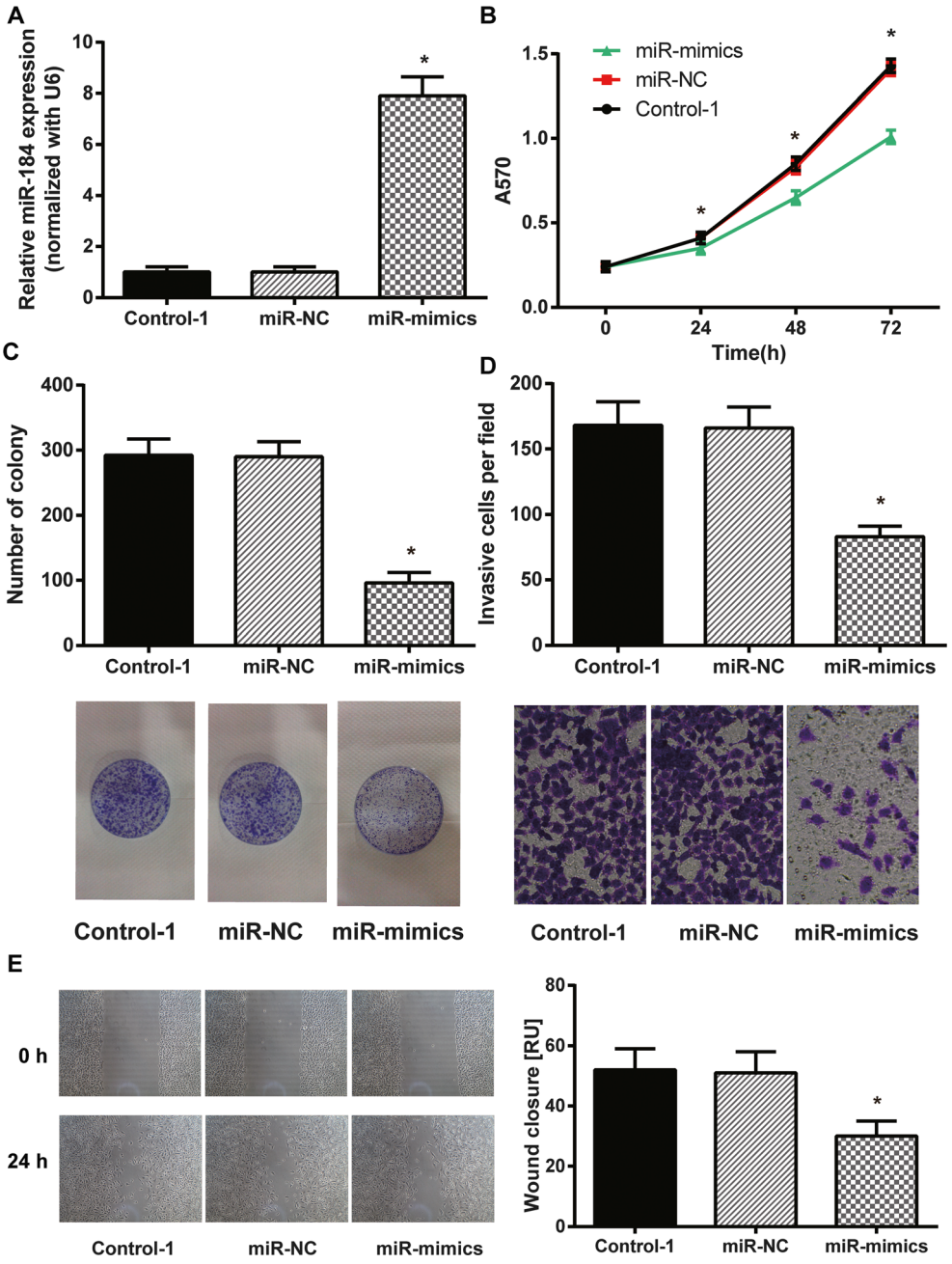

Fig. 2 Influence of miR-184 overexpression on GBM cell proliferation, invasion and migration. **a** RT-PCR was performed to analyze the miR-184 expression level in U87-MG cells after transfection. U6 served as the internal control. **b** MTT assay was conducted on U87-MG cells lipotransfected with miR-184 mimics to determine the influence of miR-184 on cell viability. **c** Colony formation assay was conducted on U87-MG cells lipotransfected with miR-184 mimics to determine the influence of miR-184 on the cell proliferation. **d** Transwell assay was conducted on U87-MG cells transfected with miR-184 mimics to determine the influence of miR-184 on cell invasiveness. E. Wound/Scratch healing assay was conducted on U87-MG cells to determine the influence of miR-184 mimics on U87-MG cells migration. ($P < 0.05$)

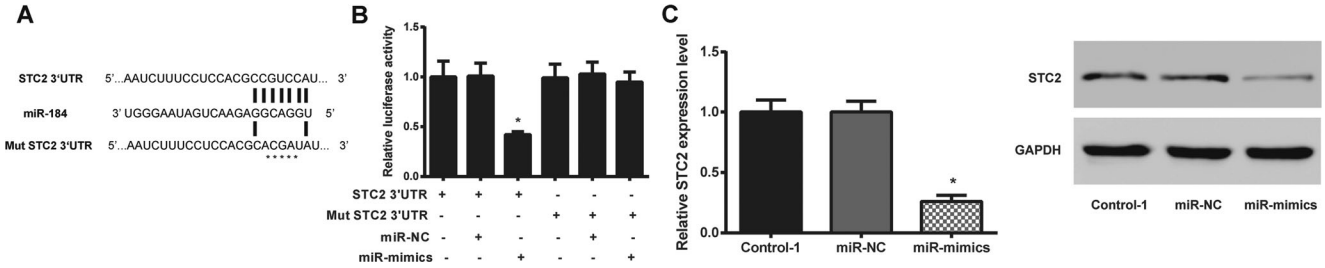

Fig. 3 MiR-184 targeted STC2. **a** The predicted miR-184 binding site in the 3'UTR of STC2 and the mutated 3'UTR of STC2. **b** MiR-184 directly targeted the wild type or mutated 3'UTR of STC2 and inhibited the luciferase activities. **c** The protein level of STC2 was detected using western blot when the U87-MG cells were transfected with miR-184 mimics. GAPDH served as the internal control. *indicates *P*-value < 0.05, compared with controls

In addition, U87-MG cells transfected with pCMV-STC2 showed substantially bigger OD value than those in the pCMV-NC group at every time point after the transfection. However, miR-184 mimics substantially reduced the viability of U87-MG cells as shown in the mimics + STC2 group (Fig. 4c).

Similarly, U87-MG cells had larger number of colonies and larger number of invasive U87-MG cells in the pCMV-STC2 group than in the pCMV-NC group. Whereas the exogenous miR-184 significantly suppressed the colony formation and invasiveness of U87-MG cells (Fig. 4d–e). The colony formation and Transwell assay results suggested that miR-184 could retard the proliferation and invasiveness of U87-MG cells by inhibiting STC2.

Not surprisingly, the wound closure rate was much bigger in the pCMV-STC2 group than in the pCMV-NC group,

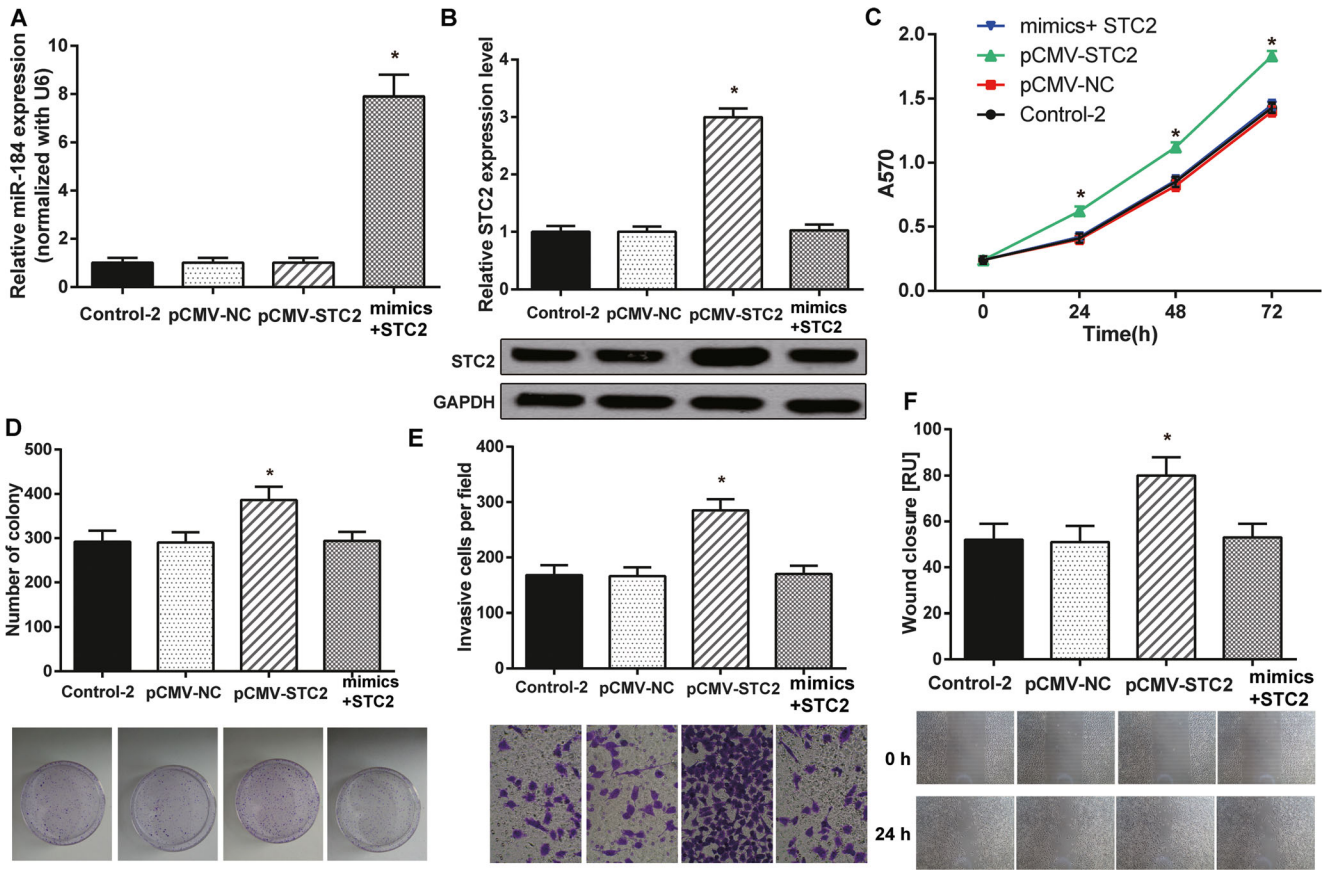

Fig. 4 MiR-184 suppressed GBM cell propagation, migration and invasiveness by targeting STC2. **a** RT-PCR was performed to analyze the miR-184 expression level in U87-MG cells after transfection. U6 served as the internal control. **b** The protein level of STC2 was detected using western blot after the U87-MG cells were transfected. GAPDH served as the internal control. **c** MTT assay was conducted on U87-MG cells lipo-transfected with miR-184 mimics or STC2 cDNAs to determine the influence of miR-184 and STC2 on cell viability. **d** Clonogenicity of U87-MG cells was determined via colony formation assay. **e** Cell invasiveness was tested by Transwell assay. **f** Cell migration was measured by wound/scratch healing assay. ($P < 0.05$)

whereas the closure rate was much smaller in the mimics + STC2 group than in the pCMV-STC2 group (Fig. 4f). The wound healing results indicated that miR-184 could reduce the cell migration by suppressing STC2.

TC2 Overexpression Induced Poor Prognosis of GBM Patients

To further investigate the effects of STC2 expression on GBM progression, we analyzed the correlation between STC2 expression and the overall survival (OS) and the disease-free survival (DFS) rate. We found that GBM patients with STC2 over-expression showed substantial poor prognosis than those with STC2 under-expression (Fig. 5).

Discussion

MiRNAs negatively regulate different genes by inducing mRNA cleavage or translation repression and perform as oncogenes or tumor suppressors. Accumulated evidence showed that miRNAs play a key role in the glioma U87-MG cell growth, including cell survival, proliferation, invasion, and migration [1, 2, 30]. MiR-184, for instance, has effects on malignant progression, as an oncogene or tumor suppressor [11, 31, 32]. As the present study indicated, miR-184 was observed to be down-regulated in both GBM tissues and U87-MG cells. Furthermore, the overexpression of miR-184 inhibited cell proliferation, invasion and migration of U87-MG cells. In addition, we further demonstrated that miR-184 suppressed the expression of STC2 by binding to 3′-UTR. The present data pointed out that the expression of miR-184 was obviously lower in GBM tissues or U87-MG cells when compared with the normal brain tissues or cells, respectively. In several types of cancers such as renal cell carcinoma [11], epithelial ovarian cancer [12], breast cancer [13] and nasopharyngeal carcinoma [33], miR-184 was claimed to be down-regulated. The present study indicated that the overexpression of miR-184 inhibited the GBM cell line U87-MG proliferation, invasion, and migration. We thus proposed that miR-184 might act as a tumor suppressor in human GBM.

STC is a secreted glycoprotein hormone first found in bony fishes, and it is secreted into blood and regulates Ca^{2+} and phosphate uptake in different organs. Human *STC2* gene, encodes a 302-amino acid-long protein, is primarily expressed in the kidney, heart, pancreas and spleen [34]. In several different human cancers, abnormal expression of STC2 has been discovered, implicating its possible involvement in cell proliferation, invasion and migration in some cancers. For instance, in cervical cancer, STC2 plays a simulating role in cancerous cell proliferation [20, 35]. Similarly, in lung cancer, hepatocellular carcinoma, nasopharyngeal carcinoma and gastric cancer, STC2 was also found involved in cancer progression [21, 23, 24, 36]. To the contrary, STC2 was regarded as a negative regulator of breast cancer cell proliferation [22]. We wonder whether the different effects of STC2 on cancer cell proliferation is due to cancer types, although further researches need to be done to confirm STC2's biological function. We here in this study, discovered that STC2 was over-expressed in GBM tissues and U87-MG cells and its suppression led to the repression of GBM cell growth. In addition to proliferation, STC2 was also shown to induce GBM cell migration and invasion. Aberrant cell migration and invasion are the primary processes involved in carcinogenesis, and they could cause

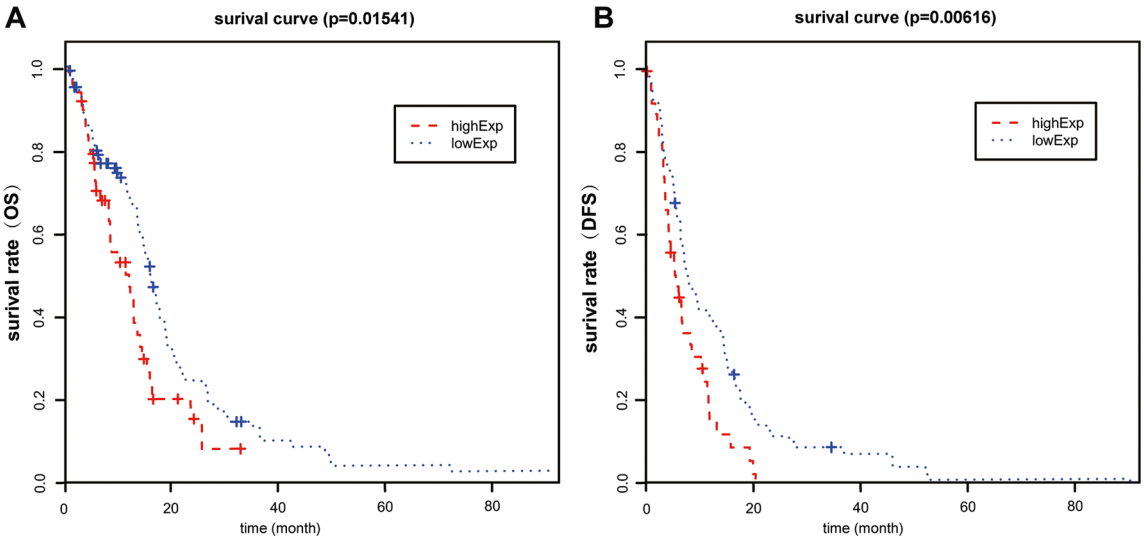

Fig. 5 High STC2 mRNA expression predicts poor survival whereas low STC2 mRNA expression indicated good survival of GBM patients. **a** The overall survival (OS) analysis of GBM patients with either high or low expression of STC2 mRNA. **b** the disease-free survival (DFS) results of GBM patients with either high or low STC2 mRNA level. The red plus signs indicate high STC2 mRNA expression, and the blue plus signs indicate low STC2 mRNA expression

distant metastasis. Cell migration, including cell development, angiogenesis, wound repair and metastasis, is known as an important feature of physiological and pathological processes [21]. Metastatic characteristics are related to various causes of death of patients with lung cancer. Another in vitro study demonstrated that *STC2* knockdown decreased cell migration and invasion [37], indicating that *STC2* expression not only affects cell proliferation but plays a key role in cancer progression by promoting cell migration and invasion.

Conclusion

In the present study, we, for the first time, demonstrated the direct targeting relationship between miR-184 and STC2 in U87-MG cells, although detailed mechanism remained to be further elucidated.

The present study pointed out that miR-184 was significantly down-regulated in U87-MG human glioma cells and tissues, whereas STC2 was up-regulated. STC2 expression was negatively correlated with miR-184 expression. The present study also identified that miR-184 down-regulated the expression of STC2 by directly binding to the 3′-UTR of STC2 mRNA within U87-MG cells. Our results showed that miR-184 was a tumor suppressor in the malignant progression of GBM and may be utilized to develop a miRNA-based therapy against GBM.

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Compliance with Ethical Standards The research has been carried out in accordance with the World Medical Association Declaration of Helsinki.

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

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