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Original Article

Fibulin-5 inhibits the cell proliferation, migration and angiogenesis in glioma

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Abstract: Glioma, especially the high-grade glioma, is the most common primary brain malignant tumor, with extremely poor prognosis. Angiogenesis has been demonstrated to play a crucial role in the growth and metastasis of glioma. Fibulin-5 is a multifunctional extracellular matrix (ECM) protein that mediates cell-cell and cell-matrix communication. Recent evidence has suggested that Fibulin-5 is involved in angiogenesis and tumor metastasis. However, the exact role of Fibulin-5 in glioma remains largely unclear. In the present study, immunohistochemical staining data showed the positive expression rate of Fibulin-5 in the normal brain tissues and low grade glioma were higher than that in high grade glioma. Moreover, the expression level of Fibulin-5 was inversely correlated with microvessel density, and Ki-67 and HIF-1α expression in human glioma tissues. We further studied the role of Fibulin-5 in the regulation of biological functions of vessel endothelial cells and glioma cells in vitro. Overexpression of Fibulin-5 significantly suppressed cell proliferation via inducing a cell cycle arrest at G1 phase in HUVEC-2C cells. Moreover, Fibulin-5 upregulation also enhanced the cell adhesion, while inhibited the migration and vessel-sprouting capacities of HUVEC-2C cells. Furthermore, co-culture of glioma U251 cells and Fibulin-5-transfected HUVEC-2C cells significantly suppressed the proliferation, migration and invasion of U251 cells. In summary, we suggest that Fibulin-5 acts as a tumor suppressor in glioma, through inhibition of vessel formation within the microenvironment of tumor tissues.

Keywords: Glioma, Fibulin-5, angiogenesis, proliferation, migration, invasion

Introduction

Glioma is the most common primary brain malignant tumor [1]. Despite advances in surgical and clinical neuro-oncology, the prognosis of high-grade glioma remains extremely poor. The microvessel density (MVD) is an independent index for the judgment of the grade of glioma, and angiogenesis has been suggested to be the most important factor for the development and progression of glioma [2-4]. Therefore, the anti-angiogenesis treatment may become a promising therapeutic strategy for glioma.

Fibulin-5, a 448 amino acid glycoprotein, is a member of the Fibulin family of extracellular matrix (ECM) proteins. It mediates interaction with cell surface integrins, including αvβ3, αvβ5 and α9β1, and thus is involved in cell adhesion [5, 6]. Moreover, it has been found to participate in the regulation of vascular formation [7, 8]. Recently, the role of Fibulin-5 in human cancers has been gradually revealed. It may act as an oncogene or tumor suppressor in different types of malignant tumors. Schiemann et al. found that overexpression of Fibulin-5 enhanced the tumorigenicity of human HT1080 fibrosarcoma cells by increasing their DNA synthesis, migration toward fibronectin, and migration through synthetic basement membranes [9]. Besides, Fibulin-5 was also found to promote nasopharyngeal carcinoma cell metastasis and correlate with poor prognosis [10]. In contrast, Fibulin-5 has also been suggested to act as a tumor suppressor in several types of human cancers, such as renal cell cancer, hepatocellular carcinoma, bladder cancer, lung cancer, and so forth [11-14]. Recently, Sheng et al. found that the expression of Fibulin-5 in glioma tissues was significantly lower than those in

normal brain tissues, and the negative expression of Fibulin-5 was significantly correlated with advanced clinical stage as well as a shorter overall survival of glioma patients. Besides, overexpression of Fibulin-5 obviously inhibited the proliferation, migration and migration of glioma U251 and U87 cells [15]. Therefore, Fibulin-5 may play a suppressive role in the malignant progression of human cancers including glioma. However, the detailed role of Fibulin-5 in the malignant progression of glioma, as well as the underlying mechanism involving angiogenesis, has not been fully uncovered.

In the study, we examined the expression of Fibulin-5 in glioma tissues and normal brain tissues, and analyzed the association of Fibulin-5 expression with the malignant progression and vessel formation in glioma. Moreover, we also aimed to reveal the role of Fibulin-5 in the regulation of biological function of vessel cells and glioma cells, including cell proliferation, cell cycle progression, adhesion, migration, as well as angiogenesis.

Methods and materials

Tissue samples

This study was approved by the legislation and ethical boards of Central South University, Changsha, China. All subjects or their caregivers have written informed consent. Total 40 glioma tissue samples were collected from November 2006 to April 2008 at the Department of Neurosurgery, Xiangya Hospital of Central South University. Among these patients with glioma, 22 are male, and 18 female; the youngest is 7 years old, and the oldest is 67 years old, with average old is 40.4. In addition, samples in grade-I is 7, grade-II 14, Grade-III 13, grade-IV 6. No preoperative chemotherapy, radiotherapy, or embolization was used in these patients. All these samples were formalin fixed and paraffin-embedded.

Immunohistochemical staining assay

The expression levels of Fibulin-5, Ki-67, Factor VIII and HIF-1α were evaluated by using immunohistochemical staining. Tissue sections of 4 μm were deparaffinized and subjected to heat-induced antigen retrieval using citrate buffer for 22 min using a microwave oven. Then the sections were incubated with antibodies against Fibulin-5 (Bioss, China), Factor VIII (ZSGB-Bio, China), HIF-1α (ZSGB-Bio, China) and Ki-67 (ZSGB-Bio, China), respectively. Subsequently, the sections were incubated with secondary antibody for 60 min at room temperature. The reaction was developed using substrate diaminobenzidine (DAB) and counterstained with hematoxylin, and visualized via optical microscopy. All tissue sections were analyzed and scored independently by three experienced pathologists. The scoring system was as follows: the percentage of positively staining cells was graded as 0 (no staining, negative), +: >0 and ≤25% of cells positive, ++: >25 and ≤75% of cells positive, +++: >75% of cells positive.

Cell culture

HUVEC-2C and U251 cell lines were purchased from the Cell Bank of Central South University. Cells were cultured in RMPI 1640 medium added with 10% fetal bovine serum (FBS, Life Technologies) at 37°C in a humidified incubator containing 5% CO_2. For co-culture assay, 24-well transwell chambers (Chemicon, CA, USA) were used. U251 cells (1×10^5) were seeded in the lower chamber, while HUVEC-2C (1×10^5) were seeded in the upper chamber.

Plasmid construction and transfection

The lenti-virus vector pGC-FU was used to construct the Fibulin-5 expressing plasmid, named pGC-FU-Fibulin-5, which was then co-transfected into 293T cells with pHelper 1.0 and Helper 2.0 using Lipofectamine 2000 (Life Technologies) according to the manufacture's instruction. Lenti-virus expressing Fibulin-5 was then used to infect HUVEC-2C cells. Western blot was conducted to select HUVEC-2C cells which stably expressed Fibulin-5.

Western blot

Cells were solubilized in cold RIPA lysis buffer. Proteins were separated with 12% SDS-PAGE, and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with TBST containing 5% skimmed milk at 37°C for 2 h. Then, the membrane was incubated with mouse anti-Fibulin-5 (1:100; Sigma Aldrich), and mouse anti-GAPDH primary antibodies (1:200; Sigma Aldrich), respectively, at

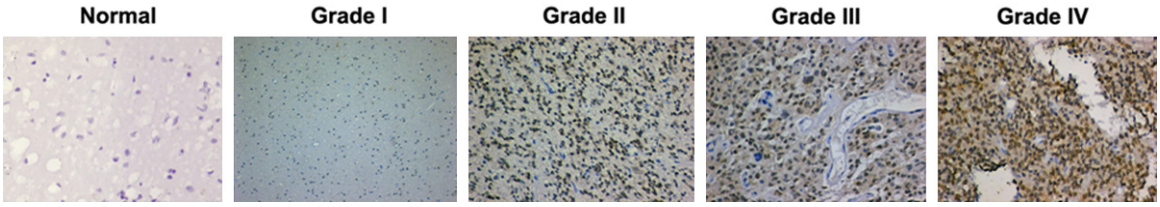

Figure 1. The Fibulin-5 expression in normal brain tissues and gliomas of different grades. Immunohistochemical staining was performed to examine the expression of Fibulin-5 in normal brain tissues and glioma samples of different grades.

Table 1. Postive expression rate of fibulin-5 in normal brain tissues and gliomas

Group	n	Fibulin-5 protein					
		-	+	++	+++	Positive rate	Mean IRS
Normal brain tissue	7	0	4	2	1	100.0%*	4.57±3.21*
Grade I-II	21	8	5	6	2	61.9%*	3.33±3.47*
Grade III-IV	19	15	4	0	0	21.1%	0.421±0.902

*P<0.05 vs. Grade III-IV.

Table 2. Postive expression of fibulin-5 in gliomas

Group	*n*	Fibulin-5 protein				
		-	+	++	+++	Positive rate
Male	22	12	4	4	2	45.5%
Female	18	11	5	2	0	38.9%
<40 year	19	11	4	4	0	42.1%
≥40 year	21	12	5	2	2	42.9%

room temperature for 2 h. After washed by PBST for 4 times with 10 min each time, the membrane was incubated with the goat anti-mouse secondary antibodies (1:5000; Sigma Aldrich) at 4°C overnight. After washed by PBST for 4 times with 10 min each time, ECL kit (Pierce Chemical, Rockford, IL, USA) was used to perform chemiluminent detection. Image-Pro plus software 6.0 was used to analyze the relative protein expression, represented as the density ratio versus GAPDH.

Cell proliferation assay

MTT assay was used to measure cell proliferation. At 72 h post-transfection, 100 μl cell suspension (5000 cells/ml) was seeded into 96-well plate, and incubated at 37°C with 5% CO_2 for 6 h, 12 h, 24 h, and 48 h, respectively. For MTT assay, the transfection medium in each well was replaced by 100 μl of fresh serum-free medium with 0.5 g/l MTT. After incubation at 37°C for 4 h, the MTT medium was removed by aspiration and 50 μl of DMSO was added to each well. After reacting for 10 min at room temperature, formazan production was detected by measurement of the optical density (OD) at 492 nm using an ELX-800 type ELISA reader (Bio-Tek, VT, USA).

Cell adhesion assay

For cell adhesion assay, each well in 24-well plate was pre-coated with collagen. After that, 20000 cells in each group were seed in each well, and cultured at 37°C, 5% CO_2 for 30 min. After washed with PBS for two times, cells were fixed and then stained for 20 min, and then rinsed by water, and dried in air. Adhesive cells were observed and numbered under the microscope (Nikon).

Cell migration assay

To determine the cell migratory capacity, cells were cultured to confluence. Wounds of approximately 1 mm width were created with a plastic scriber, and cells were washed and incubated in a serum-free medium. After wounding for 24 h, cells were incubated in a medium including 10% fetal bovine serum. Cultures at 0 and 48 h were fixed and observed under a microscope (Nikon).

Cell invasion assay

For cell invasion assay, 24-well transwell chambers pre-coated with matrigel (Chemicon, CA, USA) were used. For each group, cell suspension (200000 cells/ml) was prepared in serum free DMEM, and 500 μl of DMEM with 10% FBS was added into the lower chamber, and 200 μl of cell suspension was added into the upper

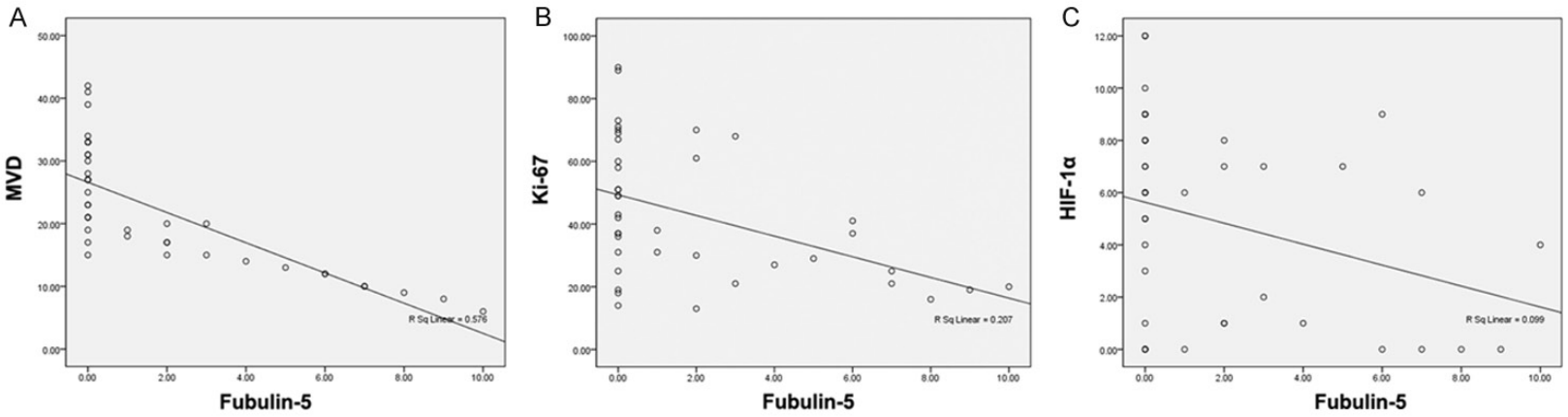

Figure 2. Correlations between Fibulin-5 expression and the expression of MVD, Ki-67, and HIF-1α. The correlations between the Fibulin-5 expression and the expression levels of MVD (A), Ki-67 (B) and HIF-1α (C) were indicated.

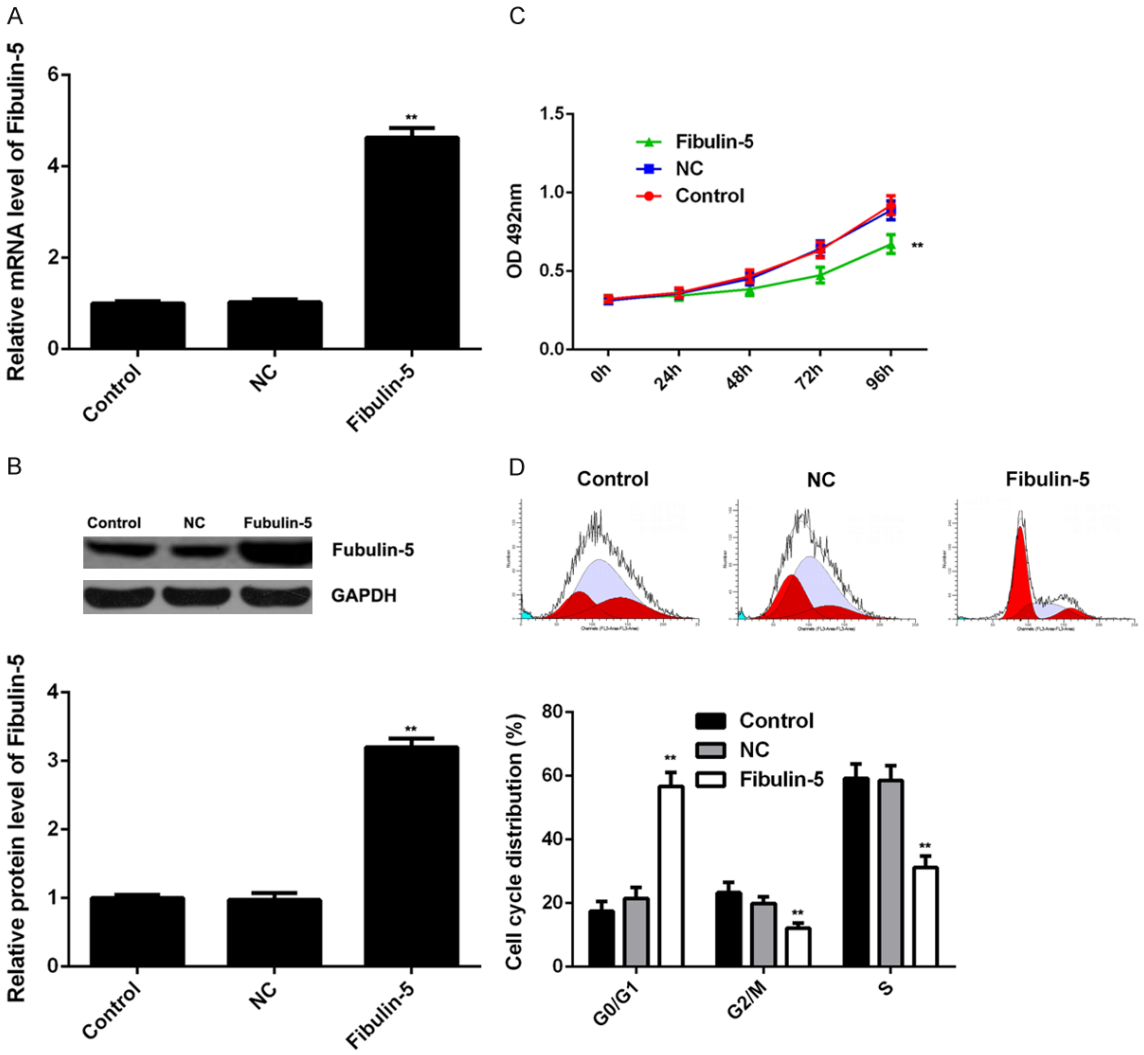

Figure 3. Inhibitory effect of Fibulin-5 on proliferation of HUVEC-2C cells. (A) Real-time RT-PCR and (B) western blot were conducted to determine the mRNA and protein level of Fubulin-5 in HUVEC-2C cells transfected with Fibulin-5 plasmid or blank vector as negative control (NC), respectively. Non-transfected HUVEC-2C cells were used as Control. After that, (C) MTT assay and (D) flow cytometry were conducted to determine the cell proliferation and cell cycle distribution, respectively. **Means P<0.01 vs. Control.

chamber. After incubation at 37°C with 5% CO_2 for 24 h, those non-migratory cells were removed, and cells through the membrane were stained for 20 min, and then rinsed by water, and dried in air, and observed under the microscope.

Statistical analysis

Continuous variables were expressed as mean ± SD. Categorical variables were expressed as percentages. SPSS 16.0 was used for statistical analyses. Correlations between Fibulin-5 expression and clinicopathologic characteristics in glioma were assessed using the χ^2 test or Fisher exact test. For in-vitro experiments, statistical analysis of differences was performed by one-way analysis of variance (ANOVA) or Student t test. P<0.05 was considered statistically significant.

Results

Expression of Fibulin-5 in glioma and normal brain tissues

We firstly examined the expression of Fibulin-5, Ki-67, Factor VIII and HIF-1α in 40 cases prima-

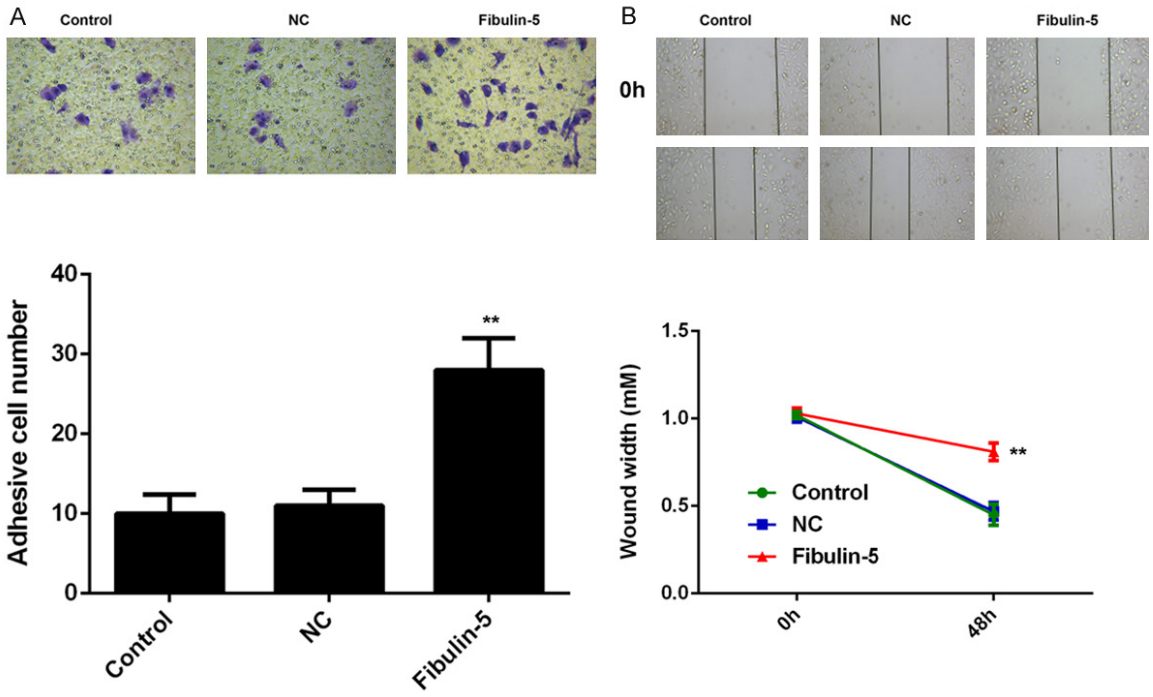

Figure 4. Inhibitory effect of Fibulin-5 on adhesion and migration of HUVEC-2C cells. A. Cell adhesion assay was conducted to examine the adhesive capacity of HUVEC-2C cells transfected with Fibulin-5 plasmid or blank vector as negative control (NC), respectively. Non-transfected HUVEC-2 cells were used as Control. B. Wound healing assay was conducted to determine the migratory capacity. **Means P<0.01 vs. Control.

ry glioma as well as 7 normal brain tissues by immunohistochemical staining. Our data showed that the positive expression rate of Fibulin-5 in the normal brain tissues and low grade glioma were higher than those in high grade glioma (**Figure 1**; **Table 1**). In addition, there was no significant difference between male and female, as well as different ages (**Table 2**). Microvessel density (MVD) was calculated according to the expression level of Factor VIII. We further analyzed the correlations between the Fibulin-5 expression and the expression levels of Ki-67, MVD and HIF-1α, respectively. As shown in **Figure 2A-C**, the expression level of Fibulin-5 was inversely correlated with MVD, Ki-67, and HIF-1α expression in human glioma (R=-0.455, -0.779, -0.320, respectively; P=0.003, 0.000, 0.044, respectively). However, although the expression level of Fibulin-5 in the normal brain tissues and the low grade glioma were higher than that in the high grade glioma, the difference was not significant (data not shown). In summary, we suggest that the positive expression rate of Fibulin-5 is inversely correlated with the malignant progression of glioma as well as the angiogenesis within the tumor microenvironment.

Inhibitory effect of Fibulin-5 on proliferation, adhesion, migration and vessel-sprouting abilities of HUVEC-2C cells

To further study the role of Fibulin-5 in the regulation of the biological functions of vessel cells, we transfected HUVEC-2C cells with Fibulin-5 plasmid or blank vector as negative control (NC), respectively. Non-transfected HUVEC-2C cells were used as Control. After transfection, the mRNA and protein expression of Fibulin-5 was significantly upregulated compared to the Control group (**Figure 3A** and **3B**). We further performed MTT assay to determine the cell proliferation in each group. As shown in **Figure 3C**, overexpression of Fibulin-5 markedly suppressed the proliferation capacity of HUVEC-2C cells compared to the Control group. As cell cycle progression plays a key role in cell proliferation, we further investigated the cell cycle distribution, and found that overexpression of Fibulin-5 induced a cell cycle arrest at G1 phase (**Figure 3D**), suggesting that the inhibitory effect of Fibulin-5 on HUVEC-2C cell proliferation is due to the cell cycle arrest at G1 stage.

We further investigated the role of Fibulin-5 in the regulation of vessel cell adhesion and

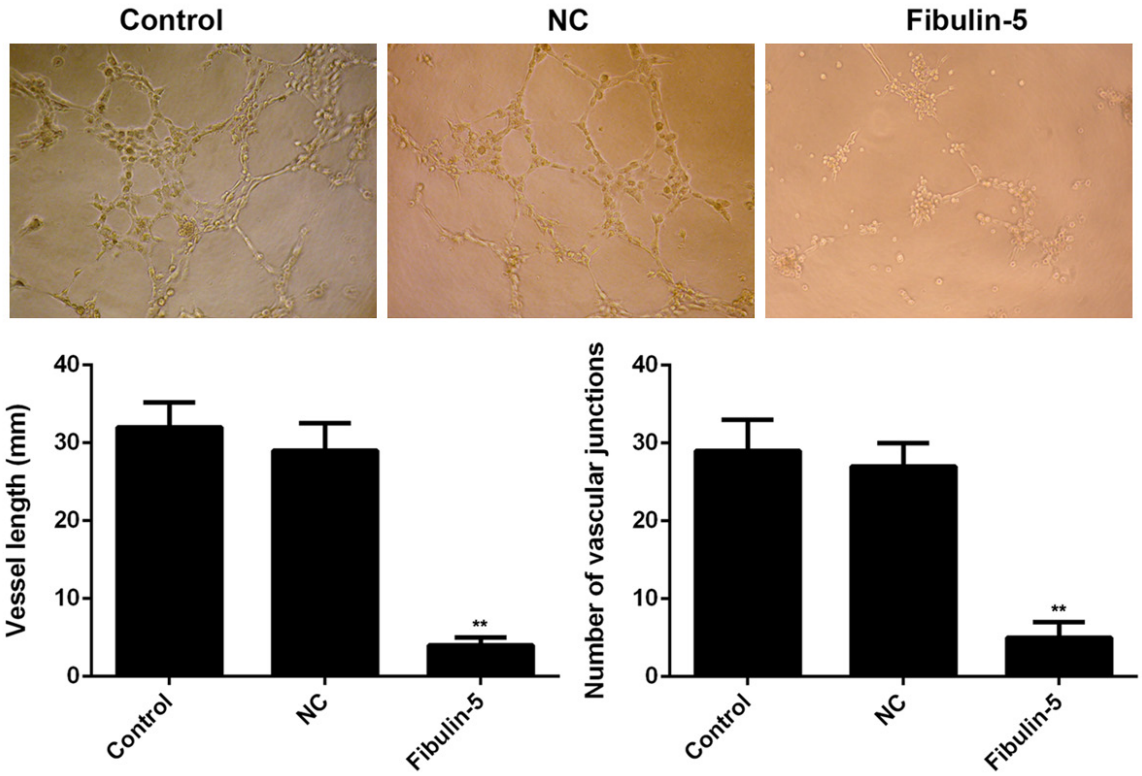

Figure 5. Inhibitory effect of Fibulin-5 on vessel-sprouting abilities of HUVEC-2C cells. HUVEC-2C cells were transfected with Fibulin-5 plasmid or blank vector as negative control (NC), respectively. Non-transfected HUVEC-2 cells were used as Control. The vessel length (mm) and the number of vascular junctions generated by HUVEC-2C cells were calculated. **Means P<0.01 vs. Control.

migration. As demonstrated in **Figure 4A** and **4B**, overexpression of Fibulin-5 significantly enhanced cell adhesion while inhibited cell migration of HUVEC-2C cells, compared to the control group. After that, we studied the role of Fibulin-5 in the regulation of the vessel-sprouting ability of vessel cells. As shown in **Figure 5**, transfection with Fibulin-5 significantly inhibited the vessel-sprouting ability of HUVEC-2C cells, compared to the control group, suggesting that Fibulin-5 has a suppressing effect on angiogenesis.

Co-culture of glioma U251 cells and Fibulin-5-transfected HUVEC-2C cells suppressed U251 cell proliferation, migration and invasion

We further investigated the effect of HUVEC-2C cells overexpressing Fibulin-5 on the malignant phenotypes of glioma cells. Glioma U251 cells and Fibulin-5-transfected HUVEC-2C cells were co-cultured to generate the microenvironment of glioma. In the control group, U251 cells were co-cultured with non-transfected HUVEC-2C cells. In the NC group, U251 cells were co-cultured with the HUVEC-2C cells transfected with blank vector. After that, we conducted MTT assay, wound healing assay, and transwell assay to determine the cell proliferation, migration and invasion of U251 cells in each group. As shown in **Figure 6A-C**, overexpression of Fibulin-5 in HUVEC-2C cells significantly suppressed the proliferation, migration and invasion of U251 cells under co-culture, when compared to the control group, respectively. There findings suggest that overexpression of Fibulin-5 in HUVEC-2C cells may have suppressive effects on glioma growth.

Discussion

Fibulin-5 is an integrin-binding ECM protein that mediates EC adhesion. Recent evidence has suggested that Fibulin-5 is involved in angiogenesis and tumor metastasis. However, the exact role of Fibulin-5 in glioma remains largely unclear. In the present study, we found that the positive expression rate of Fibulin-5 in the nor-

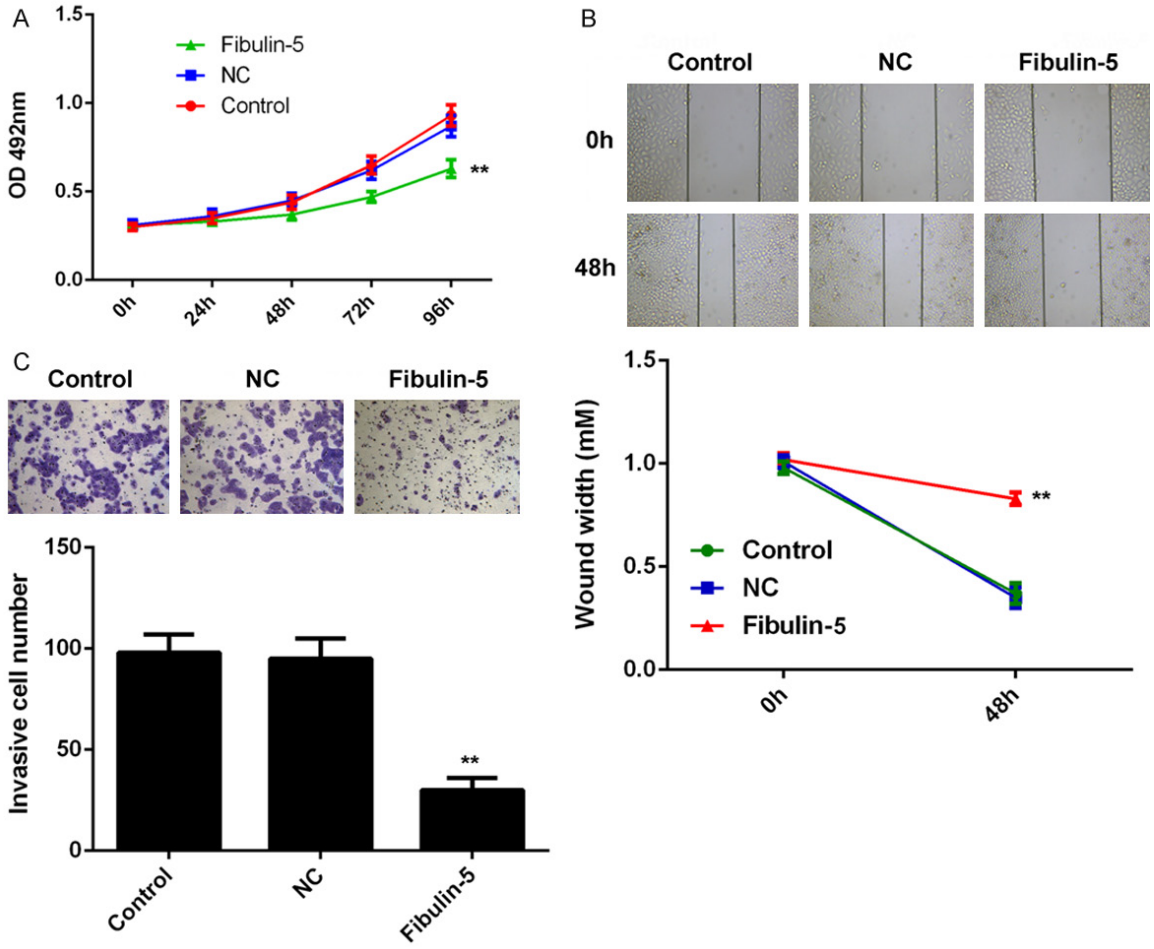

Figure 6. Co-culture of glioma U251 cells and Fibulin-5-transfected HUVEC-2C cells suppressed U251 cell proliferation, migration and invasion. Glioma U251 cells and Fibulin-5-transfected HUVEC-2C cells were co-cultured to generate the microenvironment of glioma. In the control group, U251 cells were co-cultured with non-transfected HUVEC-2C cells. In the NC group, U251 cells were co-cultured with the HUVEC-2C cells transfected with blank vector. (A) MTT assay, (B) wound healing assay and (C) transwell assay were performed to examine the proliferation, migration and invasion of U251 cells. **Means $P<0.01$ vs. Control.

mal brain tissues and low grade glioma were higher than that in high grade glioma. Moreover, the expression level of Fibulin-5 was inversely correlated with MVD as well as Ki-67 and HIF-1α expression in human glioma tissues. In-vitro investigation showed that overexpression of Fibulin-5 significantly suppressed cell proliferation via inducing a cell cycle arrest at G1 phase in HUVEC-2C cells. Moreover, Fibulin-5 upregulation also enhanced the adhesion, but inhibited the migration and vessel-sprouting capacities of HUVEC-2C cells. Furthermore, co-culture of glioma U251 cells and Fibulin-5-transfected HUVEC-2C cells suppressed the proliferation, migration and invasion of U251 cells.

Fibulin-5 is secreted by various cell types, including ECs, vascular smooth muscle cells, and fibroblasts, contributing to the formation of elastic fibers by binding to tropoelastin and fibrillin-1, and to cross-linking enzymes, aiding elastic fiber assembly [16]. It has been demonstrated that Fibulin-5 is important for normal embryonic development and organogenesis. For instance, mice deficient in Fibulin-5 exhibit systemic elastic fiber defects with manifestations of loose skin, emphysematous lung, tortuous aorta, and genital prolapse [17, 18]. Recently, Fibulin-5 has been suggested to be associated with the inhibition of tumorigenesis through its control of cell proliferation, motility and angiogenic sprouting. Wlazlinski et al. found that Fibulin-5 was frequently downregulated in prostate cancer [19]. In our study, we showed that the positive expression rate of Fibulin-5 was significantly lower in high grade

glioma, when compared to low grade glioma and normal brain tissues. Recently, Sheng et al. showed similar findings that the expression of Fibulin-5 in glioma tissues was significantly lower than those in normal brain tissues. Besides, they also found that the negative expression of Fibulin-5 was significantly correlated with advanced clinical stage (grade III+IV) as well as a shorter overall survival of glioma patients, and demonstrated that Fibulin-5 was an independent factor for predicting overall survival of glioma patients [15]. Based on these findings and ours, we suggest that downregulation of Fibulin-5 contributes to the malignant progression of glioma, and is tightly associated with poor prognosis of glioma patients.

It has been well-established that angiogenesis plays a key role in tumor progression [20]. Therefore, anti-angiogenesis becomes an important and effective strategy for the treatment of human cancers including glioma. For instance, Li et al. showed that the high efficacy of lidamycin and the synergistic effects of lidamycin plus temozolomide against glioma are mediated, at least in part, by the potentiated anti-angiogenesis [20]. Moreover, various factors have been found to regulate angiogenesis in glioma, and thus may be used as promising therapeutic targets or candidates. Huang et al. found that VE-statin/Egfl7 siRNA inhibited angiogenesis in malignant glioma in vitro [21]. In addition, meriolins are a new class of cyclin-dependent kinase inhibitors, and can suppress cell proliferation and neo-angiogenesis in glioma [22]. In this study, we showed that Fibulin-5 had suppressive effects on the proliferation, migration and vessel-sprouting capacities of HUVEC-2C cells, suggesting that Fibulin-5 can inhibit angiogenesis in vitro. In fact, Sullivan et al. have reported that Fibulin-5 function as an inhibitor molecule in initial sprouting and/or migration of ECs [23]. They showed a significant increase in vascular invasion, with no increase in fibroblast migration, into sponges removed from Fibulin-5 knockout (KD) mice compared with wild-type mice. Besides, they suggested that VEGF and angiopoietins might be involved in Fibulin-5-mediated angiogenesis [23].

In addition, the dual roles of Fibulin-5 have been suggested in a variety of human cancers. For instance, Tu et al. found that Fibulin-5 inhibited the migration and invasion of hepatocellular carcinoma cells by inhibition of matrix metalloproteinase-7 expression [12]. On the contrary, Shi et al. reported that the high expression of Fibulin-5 was associated with the malignant clinicopathologic parameters in gastric cancer and Fibulin-5 knockdown inhibited cell proliferation and invasion in gastric cancer cells [24]. Therefore, the dual roles of Fibulin-5 seem to be tumor specific. Recently, Sheng et al. found that overexpression of Fibulin-5 markedly inhibited the proliferation, migration and invasion of glioma U251 and U87 cells [15]. In our study, we showed that overexpression of Fibulin-5 in HUVEC-2C cells significantly inhibited U251 cell proliferation, migration and invasion under co-culture, suggesting that inhibition of angiogenesis within the tumor microenvironment may contribute to the suppression of tumor growth and metastasis.

In summary, our study revealed a crucial role of Fibulin-5 in the regulation of angiogenesis within the glioma microenvironment, and suggests that Fibulin-5 may become a potential candidate for the treatment of glioma.

Disclosure of conflict of interest

None.

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