

FRAT1 promotes the angiogenic properties of human glioblastoma cells via VEGFA

BIAO YANG^{1*}, DONG LIU^{1*}, YE-QING REN^{1*}, YAN-QI SUN¹, JIAN-PING ZHANG²,
XIAO-GANG WANG¹, YONG-QIANG WU¹, SHU-LE WANG¹, SHI-HAO GUO¹ and GENG GUO¹

¹Department of Neurosurgery, The First Hospital of Shanxi Medical University, Taiyuan, Shanxi 030001;

²Department of Neurosurgery, The Affiliated Liuzhou People's Hospital of Guangxi Medical University, Liuzhou, Guangxi Zhuang Autonomous Region 545006, P.R. China

Received November 2, 2021; Accepted December 16, 2021

DOI: 10.3892/mmr.2022.12611

Abstract. Glioblastoma is a common central nervous system tumor and despite considerable advancements in treatment patient prognosis remains poor. Angiogenesis is a significant prognostic factor in glioblastoma, anti-angiogenic treatments represent a promising therapeutic approach. Vascular endothelial growth factor A (VEGFA) is a predominant regulator of angiogenesis and mounting evidence suggests that the Wnt signaling pathway serves a significant role in tumor angiogenesis. As a positive regulator of the Wnt/ β -catenin signaling pathway, frequently rearranged in advanced T-cell lymphomas-1 (FRAT1) is highly expressed in human glioblastoma and is significantly associated with glioblastoma growth, invasion and migration, as well as poor patient prognosis. Bioinformatics analysis demonstrated that both VEGFA and FRAT1 were highly expressed in most tumor tissues and associated with prognosis. However, whether and how FRAT1 is involved in angiogenesis remains to be elucidated. In the present study, the relationship between FRAT1 and VEGFA in angiogenesis was investigated using the human glioblastoma U251 cell line. Small interfering RNAs (siRNAs) were used to silence FRAT1 expression in U251 cells, and the mRNA and protein expression levels of VEGFA, as well as the concentration of VEGFA in U251 cell supernatants, were determined using reverse transcription-quantitative PCR, western blotting and ELISA. A tube formation assay was conducted to assess angiogenesis. The results demonstrated that siRNA knockdown significantly

decreased the protein expression levels of FRAT1 in U251 cells and markedly decreased the mRNA and protein expression levels of VEGFA. Furthermore, the concentration of VEGFA in the cell supernatant was significantly reduced and angiogenesis was suppressed. These results suggested that FRAT1 may promote VEGFA secretion and angiogenesis in human glioblastoma cells via the Wnt/ β -catenin signaling pathway, supporting the potential use of FRAT1 as a promising therapeutic target in human glioblastoma.

Introduction

Glioblastoma is the most common malignant primary tumor of the central nervous system (CNS), accounting for 48.3% of all malignant primary CNS tumors (1). The 5-year relative survival rate of patients with glioblastoma is ~6.8% (1). Even with the use of treatments such as maximal safe surgical resection, radiotherapy and systemic chemotherapy with temozolomide, patient prognosis remains poor. The median progression-free and overall survival times from diagnosis are 6.2-7.5 and 14.6-16.7 months, respectively (2). Research has suggested that angiogenesis is a contributing factor to the growth and malignancy of glioblastoma. Angiogenesis, the budding of capillaries from pre-existing vasculature, occurs in both embryonic development and certain physiological and pathological conditions, such as menstruation, wound healing, diabetic retinopathy, rheumatoid arthritis and psoriasis, as well as in human gliomas (3). In glioblastoma, angiogenesis is the result of an imbalance between pro- and anti-angiogenic factors (4). Since 1971 when Folkman (5) proposed that tumor growth depended on blood vessel growth, VEGF has been proven to be an important angiogenic factor affecting health and disease. Accumulating evidence has indicated that vascular endothelial growth factor A (VEGFA) is highly upregulated in glioblastoma (6). The expression levels of VEGFA and its receptors (VEGFR-1 and -2) in glioblastoma are positively associated with tumor grade and negatively associated with survival time (7). The Wnt/ β -catenin signaling pathway has also been reported to serve an important role in the regulation of VEGFA secretion and angiogenesis in malignancies, such as colon cancer (8-11). Although the mechanism of VEGFA's

Correspondence to: Dr Geng Guo, Department of Neurosurgery, The First Hospital of Shanxi Medical University, 85 Jiefangnan Road, Taiyuan, Shanxi 030001, P.R. China
E-mail: guogeng973@163.com

*Contributed equally

Key words: frequently rearranged in advanced T-cell lymphomas-1, glioblastoma, angiogenesis, Wnt/ β -catenin signaling pathway, vascular endothelial growth factor A

role in these malignancies has been studied extensively, little is known about the role of VEGFA in glioblastoma angiogenesis.

The frequently rearranged in advanced T-cell lymphomas-1 (FRAT1) gene, located at the human chromosome 10q24.1 region, encodes a 29-kDa protein comprising 279 amino acids (12) and is primarily cloned from mouse T-cell lymphomas (13). FRAT1 positively regulates the Wnt/ β -catenin signaling pathway by inhibiting the activity of GSK-3 β toward β -catenin (14-16). Aberrant activation of FRAT1 promotes Wnt/ β -catenin signaling pathway dysregulation and FRAT1 is highly upregulated in a variety of human cancers (12,17-20). Our previous study demonstrated that FRAT1 is an important factor for the development of gliomas, as well as for predicting patient prognosis. Moreover, the expression of FRAT1 is positively correlated with pathological grade and angiogenesis in patients with glioma (21). Therefore, the Wnt/ β -catenin signaling pathway may serve as a positive inducer of angiogenesis in human glioma and FRAT1 may serve a significant role in this process.

To verify and elucidate this hypothesis in the present study, the glioblastoma and low-grade glioma (GBMLGG) RNA-sequencing (RNA-seq) data from The Cancer Genome Atlas (TCGA) database was used to compare the differences in FRAT1 and VEGFA expression between tumor tissues and normal samples. The potential mechanism by which VEGFA and FRAT1 modulated the occurrence and development of GBMLGG was explored and discussed. To verify this analysis, the association between FRAT1 and VEGFA was investigated using reverse transcription-quantitative PCR (RT-qPCR), western blotting, ELISA and tube formation assays.

Materials and methods

RNA-seq data and bioinformatics analysis. TCGA project (<https://genome-cancer.ucsc.edu/>) was used to collect RNA-seq data (22) and clinical information from 696 pan-cancer samples and 1,157 normal samples. According to the expression of VEGFA and FRAT1 in the samples, samples were divided into high expression and low expression groups by the median of VEGFA and FRAT1 expression, respectively. The downloaded data format was level 3 HiSeq-fragments per kilobase per million, which was converted into transcripts per million format for subsequent analysis. All procedures performed in the present study were in accordance with the Declaration of Helsinki (23).

Enrichment analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG; <https://www.genome.jp/kegg/pathway.html>), WikiPathway (WP; <https://www.wikipathways.org/index.php/WikiPathways>) and Gene Ontology (GO; <http://geneontology.org>) databases (24) were analyzed with clusterProfiler (25). Gene Set Enrichment Analysis (GSEA) was performed using GSEA 2.0 (26).

Cell culture and transfection. Human U251 glioblastoma cell lines and primary HUVECs were purchased from the American Type Culture Collection and used for subsequent experimentation. The use of primary HUVECs was approved by the ethics committee of The First Hospital of Shanxi

Medical University [approval no. (2021) Y10]. U251 cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.). HUVECs were cultured in endothelial cell basal medium supplemented with a Growth Medium Supplement Pack (both purchased from PromoCell GmbH). Both cell types were incubated at 37°C (5% CO₂) in a humidified incubator. The density at transfection was 2x10⁶ cells/ml. The medium was replaced every 2 days and the cells were passaged twice weekly.

To silence the expression of FRAT1, U251 cells were seeded into 6-well culture plates and transfected with siRNAs directly targeting FRAT1 (siFRAT1), or the corresponding negative control (NC) siRNA (siNC). Both siFRAT1 and siNC were transfected into cells using LipofectamineTM RNAiMAX Reagent (cat. no. 13778030; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The concentration of siRNA and siNC transfected was 40 nM. The sequence of siNC was 5'-UUCUCCGAA CGUGUCACGU-3'. The sequence of siFRAT1 was 5'-GCA GUUACGUGCAAAGCUU-3'. Transfection efficiency was assessed via RT-qPCR. Untransfected U251 cells were used as a control. The transfection was performed according to the manufacturer's instructions. The cells were collected after transfection at 37°C for 48 h.

VEGFA ELISA. A VEGFA ELISA was performed to quantify the secretion of VEGFA into the supernatants of non-transfected, siFRAT1 and siNC U251 cells. Cells were cultured overnight in DMEM containing 10% FBS to achieve 80-90% confluency. The culture medium were removed and the cells were washed with DMEM, prior to culturing in fresh media containing 2% FBS for 48 h. The Human VEGF ELISA kit (cat. no. EHC108) was purchased from Neobioscience Technology Co., Ltd. Supernatants were collected and the VEGFA concentration was determined using the VEGFA ELISA kit according to manufacturer's protocol.

Tube formation assay. Untransfected, siFRAT1 and siNC U251 cells were cultured in complete media (10% FBS) at 37°C for 48 h, after which the media was replaced with serum-free media at 37°C for 24 h. Subsequently, 2x10⁴ HUVECs (in 100 μ l) were seeded into a 96-well plate precoated (performed at 37°C for 45 min) with solidified Matrigel (BD Biosciences) and were incubated with the cell supernatants from U251 cells at 37°C for 6 h. Relative changes in tube length among the three groups were visualized using the Cellomics ArrayScan VT1 Readers (Cellomics, Inc.; Thermo Fisher Scientific, Inc.).

Western blotting. In the present study, non-transfected, siFRAT1 and siNC U251 cells were harvested and lysed on ice for 30 min using RIPA lysis buffer (cat. no. P0013C; Beyotime Institute of Biotechnology, Inc.). The lysed cells were centrifuged at 12,000 x g at 4°C for 20 min and the protein concentration was determined using a BCA Protein Assay Kit (Pierce; Thermo Fisher Scientific, Inc.). Total protein (20 μ l protein/lane) was subjected to SDS-PAGE using a 12% gel and the separated proteins were transferred onto a

polyvinylidene fluoride membrane (Thermo Fisher Scientific, Inc.). The membrane was blocked using TBS with 0.1% Tween-20 (TBST; pH 7.5) containing 5% nonfat dry milk for 1 h at room temperature. Membranes were subsequently incubated overnight at 4°C with the following primary antibodies: Anti-VEGFA (1:1,000; cat. no. 66828-1-Ig; ProteinTech Group, Inc.) and anti- β -actin (1:1,000; cat. no. 3700S; Cell Signaling Technology, Inc.). Following the primary incubation, the membrane was incubated with a HRP-conjugated secondary antibody (1:10,000; cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h. Then, the membrane was washed three times with TBST. Subsequently, protein expression levels were visualized using an Novex™ enhanced chemiluminescence detection solution (cat. no. WP20005; Pierce; Thermo Fisher Scientific, Inc.).

RT-qPCR. The expression levels of FRAT1 mRNA in U251 cells were assessed using RT-qPCR. Total RNA was extracted from non-transfected, siFRAT1 and siNC U251 cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RT-qPCR was performed using the RNA PCR Kit (avian myeloblastosis virus) version 3.0 (SYBR Green; Takara Bio, Inc.) according to manufacturer's protocol and the following primers were used for qPCR: Human FRAT1 forward (F), 5'-GCCCTGTCTAAA GTGTATTTTCAG-3' and reverse (R), 5'-CGCTTGAGTAGG ACTGCAGAG-3' (27); human VEGFA F, 5'-AGGGCAGAA TCATCACGAAGT-3' and R, 5'-AGGGTCTCGATTGGA TGGCA-3'; and β -actin F, 5'-GAGCTGCGTGTGGCTCCC-3' and R, 5'-CCAGAGGCGTACAGGGATAGCA-3'. The qPCR thermocycling conditions were as follows: 95°C for 1 min; 40 cycles at 95°C for 30 sec, 60°C for 10 sec and 72°C for 20 sec; and final extension at 95°C for 15 sec, 60°C for 15 sec and 95°C for 15 sec. The qPCR products were quantified using the $2^{-\Delta\Delta C_q}$ method (28) and β -actin was used as the internal control for mRNA expression.

Statistical analysis. Each experiment was repeated three times. Statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad Software, Inc.) and data are presented as the mean \pm standard deviation. Statistical differences among ≥ 3 groups were determined using a one-way ANOVA followed by a Tukey's post hoc test. The TCGA data statistical analyses were performed using R (version 3.6.3) (29). Wilcoxon rank sum test was used for continuous variables. The χ^2 test and Fisher's exact test were used for categorical variables. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Clinical characteristics. The clinical and demographic characteristics of the participants are summarized in Table I and Table II. A total of 696 individuals underwent assessment for the study. According to the expression of mRNA, individuals were divided into high expression and low expression groups. Overall, it was determined that certain groups of individuals exhibited statistically significant differences in terms of demographic or clinical characteristics between the high expression group and low expression group

($P < 0.05$). Subsequently, a multivariate analysis with the Cox regression model was performed. The results indicated that FRAT1 and VEGFA expression, World Health Organization grade, primary therapy outcome and age are significantly independently associated (Table III). Multivariate analysis demonstrated that FRAT1 expression levels are a significant independent risk factor for overall survival in GBMLGG, but VEGFA expression levels are not statistically significant.

mRNA expression status of FRAT1 and VEGFA. As evaluated by the Wilcoxon rank-sum test, the expression levels of FRAT1 and VEGFA were significantly higher in tumor tissues compared with normal tissues (Fig. 1A and B), especially for GBMLGG (Fig. 1C and D; $P < 0.001$). These results therefore indicated that the expression of FRAT1 and VEGFA was associated.

FRAT1 and VEGFA-related functional enrichment analysis. GO and KEGG enrichment analysis of genes with associated expression revealed various overrepresented terms in the following three main functional groups: 'cellular component', 'biological process' and 'molecular function'. Out of the three categories, FRAT1 expression patterns (Fig. 2) were mainly related to 'receptor ligand activity', 'antigen binding', 'leukocyte migration', 'humoral immune response', 'collagen-containing extracellular matrix', 'blood microparticle', 'immunoglobulin complex' and 'extracellular matrix structural constituent' among others, with numerous terms being associated with blood cells and angiogenesis. The KEGG analysis demonstrated that numerous terms, such as 'cytokine-cytokine receptor interaction', 'PI3K-Akt signaling pathway' and 'focal adhesion' were highly enriched in differentially expressed FRAT1. VEGFA expression also enriched numerous GO terms and KEGG pathways, including 'leukocyte migration', 'external side of plasma membrane', 'receptor ligand activity' and 'hematopoietic cell lineage', which refer to angiogenesis and immunity (Fig. 3). GSEA analysis of FRAT1 single gene association further explored the pathway process involved in FRAT1 expression (Fig. 4). The results demonstrated that certain pathways, such as 'WP_SIGNALING_PATHWAYS_IN_GLIOMASTOMA', 'KEGG_ENDOCYTOSIS', 'WP_CIRCADIAN_RHYTHM_RELATED_GENES', 'KEGG_WNT_SIGNALING_PATHWAY' and 'KEGG_PHOSPHATIDYLINOSITOL_SIGNALING_SYSTEM', were enriched and related to the regulation of angiogenesis. The aforementioned results indicated that the functions of FRAT1 and VEGFA overlap in certain functional groups and that there may be an association between them.

FRAT1 knockdown significantly downregulates FRAT1 expression levels in human U251 glioblastoma cells. To further validate the aforementioned results, following siRNA knockdown, the relative FRAT1 mRNA expression levels were detected using RT-qPCR. The untransfected group and siNC group demonstrated no significant difference in FRAT1 mRNA expression levels. However, following FRAT1 knockdown, FRAT1 was significantly downregulated in U251 cells compared with the untransfected and NC groups (Fig. 5A; $P < 0.001$). These results indicated successful silencing of FRAT1 in U251 cells.

Table I. Clinical and demographic characteristics of patients with high and low FRAT1 mRNA expression levels.

Characteristic	Low FRAT1 expression levels	High FRAT1 expression levels	P-value
n	348	348	
WHO grade, n (%)			<0.001
G2	83 (13.1)	141 (22.2)	
G3	99 (15.6)	144 (22.7)	
G4	147 (23.1)	21 (3.3)	
IDH status, n (%)			<0.001
WT	202 (29.4)	44 (6.4)	
Mut	138 (20.1)	302 (44.0)	
1p/19q codeletion, n (%)			<0.001
Codel	56 (8.1)	115 (16.7)	
Non-codel	285 (41.4)	233 (33.8)	
Primary therapy outcome, n (%)			0.023
PD	48 (10.4)	64 (13.9)	
SD	57 (12.3)	90 (19.5)	
PR	13 (2.8)	51 (11.0)	
CR	53 (11.5)	86 (18.6)	
Gender, n (%)			0.146
Female	139 (20.0)	159 (22.8)	
Male	209 (30.0)	189 (27.2)	
Race, n (%)			0.140
Asian	7 (1.0)	6 (0.9)	
Black or African American	22 (3.2)	11 (1.6)	
White	313 (45.8)	324 (47.4)	
Age, n (%)			<0.001
≤60	243 (34.9)	310 (44.5)	
>60	105 (15.1)	38 (5.5)	
Histological type, n (%)			<0.001
Astrocytoma	87 (12.5)	108 (15.5)	
Glioblastoma	147 (21.1)	21 (3.0)	
Oligoastrocytoma	43 (6.2)	91 (13.1)	
Oligodendroglioma	71 (10.2)	128 (18.4)	
OS event, n (%)			<0.001
Alive	172 (24.7)	252 (36.2)	
Dead	176 (25.3)	96 (13.8)	
DSS event, n (%)			<0.001
Alive	174 (25.8)	257 (38.1)	
Dead	161 (23.9)	83 (12.3)	
PFI event, n (%)			<0.001
Alive	143 (20.5)	207 (29.7)	
Dead	205 (29.5)	141 (20.3)	
Age, median (IQR)	52 (39, 62)	39 (32, 51.25)	<0.001

FRAT1, frequently rearranged in advanced T-cell lymphomas-1; WHO, World Health Organization; IDH, isocitrate dehydrogenase; WT, wild type; Mut, mutant; PD, progressive disease; SD, stable disease; PR, partial response; CR, complete response; OS, overall survival; DSS, disease free survival; PFI, progression-free interval; IQR, interquartile range.

FRAT1 knockdown suppresses VEGFA expression in human U251 glioblastoma cells. VEGFA is a major pro-angiogenic factor (5). To investigate the relationship between FRAT1

and VEGFA, the mRNA and protein expression levels of VEGFA were detected in untransfected, siFRAT1 and siNC U251 cells and the concentration of VEGFA was measured in

Table II. Clinical and demographic characteristics of patients with high and low VEGFA mRNA expression levels.

Characteristic	Low VEGFA expression levels	High VEGFA expression levels	P-value
n	348	348	
WHO grade, n (%)			<0.001
G2	177 (27.9)	47 (7.4)	
G3	128 (20.2)	115 (18.1)	
G4	9 (1.4)	159 (25.0)	
IDH status, n (%)			<0.001
WT	39 (5.7)	207 (30.2)	
Mut	307 (44.8)	133 (19.4)	
1p/19q codeletion, n (%)			<0.001
Codel	108 (15.7)	63 (9.1)	
Non-codel	240 (34.8)	278 (40.3)	
Primary therapy outcome, n (%)			0.013
PD	60 (13.0)	52 (11.3)	
SD	104 (22.5)	43 (9.3)	
PR	38 (8.2)	26 (5.6)	
CR	97 (21.0)	42 (9.1)	
Gender, n (%)			0.592
Female	153 (22.0)	145 (20.8)	
Male	195 (28.0)	203 (29.2)	
Race, n (%)			0.072
Asian	3 (0.4)	10 (1.5)	
Black or African American	13 (1.9)	20 (2.9)	
White	322 (47.1)	315 (46.1)	
Age, n (%)			<0.001
≤60	313 (45)	240 (34.5)	
>60	35 (5.0)	108 (15.5)	
Histological type, n (%)			<0.001
Astrocytoma	125 (18.0)	70 (10.1)	
Glioblastoma	9 (1.3)	159 (22.8)	
Oligoastrocytoma	95 (13.6)	39 (5.6)	
Oligodendroglioma	119 (17.1)	80 (11.5)	
OS event, n (%)			<0.001
Alive	270 (38.8)	154 (22.1)	
Dead	78 (11.2)	194 (27.9)	
DSS event, n (%)			<0.001
Alive	271 (40.1)	160 (23.7)	
Dead	71 (10.5)	173 (25.6)	
PFI event, n (%)			<0.001
Alive	221 (31.8)	129 (18.5)	
Dead	127 (18.2)	219 (31.5)	
Age, median (IQR)	39.5 (32.0, 50.0)	53 (38.0, 63.0)	<0.001

FRAT1, frequently rearranged in advanced T-cell lymphomas-1; WHO, World Health Organization; IDH, isocitrate dehydrogenase; WT, wild type; Mut, mutant; PD, progressive disease; SD, stable disease; PR, partial response; CR, complete response; OS, overall survival; DSS, disease free survival; PFI, progression-free interval; IQR, interquartile range.

the cell supernatant. VEGFA protein expression levels were detected via western blotting. The results demonstrated that VEGF protein expression levels were markedly reduced in

siFRAT1 U251 cells compared with untransfected and siNC U251 cells (Fig. 5B). Subsequently, the effects of FRAT1 knockdown on the VEGFA mRNA expression level were

Table III. Univariate and multivariate Cox regression model of prognosis for VEGFA and FRAT1 in patients with GBMLGG.

Characteristic	Total (n)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
WHO grade (G3 and G4 vs. G2)	612	5.893 (4.015-8.648)	<0.001	2.608 (1.600-4.251)	<0.001
1p/19q codeletion (non-codelet vs. codelet)	663	4.635 (2.963-7.251)	<0.001	1.424 (0.776-2.612)	0.254
Primary therapy outcome (PR and CR vs. PD and SD)	443	0.204 (0.114-0.365)	<0.001	0.232 (0.124-0.436)	<0.001
IDH status (Mut vs. WT)	660	0.102 (0.077-0.135)	<0.001	0.457 (0.256-0.816)	0.008
Gender (male vs. female)	669	1.230 (0.955-1.585)	0.109		
Age (>60 vs. ≤60)	669	4.716 (3.609-6.161)	<0.001	3.467 (2.109-5.698)	<0.001
Histological type (glioblastoma, oligoastrocytoma and oligodendroglioma vs. astrocytoma)	669	1.470 (1.096-1.971)	0.010	0.928 (0.592-1.454)	0.744
FRAT1 (high vs. low expression)	669	0.287 (0.219-0.374)	<0.001	0.388 (0.242-0.621)	<0.001
VEGFA (high vs. low expression)	669	4.488 (3.392-5.940)	<0.001	1.291 (0.793-2.102)	0.304

FRAT1, frequently rearranged in advanced T-cell lymphomas-1; WHO, World Health Organization; IDH, isocitrate dehydrogenase; WT, wild type; Mut, mutant; PD, progressive disease; SD, stable disease; PR, partial response; CR, complete response.

assessed via RT-qPCR. The results demonstrated that FRAT1 knockdown significantly inhibited VEGFA mRNA expression in siFRAT1 U251 cells compared with the untransfected and siNC U251 cells (Fig. 5C; $P<0.001$). The effects of FRAT1 knockdown on VEGFA secretion were further confirmed by ELISA. Silencing of FRAT1 significantly downregulated VEGFA levels in the supernatants of siFRAT1 U251 cells compared with those of untransfected and siNC-U251 cells (Fig. 5D; $P<0.001$). The ELISA results were consistent with the results of the RT-qPCR and western blotting. Furthermore, the aforementioned results displayed no significant difference between the untransfected and siNC groups. These results indicated that FRAT1 expression is associated with VEGFA expression in U251 cells.

FRAT1 knockdown inhibits angiogenesis in HUVECs cultured with U251 cell supernatants. Our previous study demonstrated that high FRAT1 expression is positively correlated with angiogenesis in human glioma tissues (21), which suggests that FRAT1 knockdown may be a promising method for inhibiting glioma angiogenesis. However, the associated underlying mechanism remains unclear. The effects of FRAT1 on angiogenesis were confirmed using a tube formation assay, to determine whether FRAT1 knockdown in human U251 glioblastoma cells impaired the angiogenesis of HUVECs. In the present study, HUVECs were cultured at 37°C for 6 h with supernatants collected from untransfected, siFRAT1 and siNC U251 cells, after which tube formation assays were conducted. For HUVEC angiogenic ability, the untransfected and siNC groups displayed no significant differences. However, the supernatants from the siFRAT1 U251 cells markedly impaired HUVEC tube formation ability (Fig. 6A) and relative changes in tube length were significantly decreased in the siFRAT1 group, compared with the untransfected and siNC groups (Fig. 6B; $P<0.001$). These results indicated that FRAT1 knockdown in U251 cells may significantly inhibit *in vitro* angiogenesis.

Discussion

Glioblastoma is one of the most highly vascularized tumors that is characterized by microvascular proliferation and endothelial cell hyperplasia (30). Patients with glioblastoma have a poor prognosis and glioblastoma tumor growth and malignancy are significantly correlated with angiogenesis (30). The postoperative survival rates of patients with high tumor microvascular density are shorter than those with low microvascular density (31). Since gliomas exist in a dormant state when exceeding 1-2 mm³ without neovascularization (32), angiogenesis is a prerequisite to overcoming nutrient and oxygen deficiency and removing waste products. Notably, these newly formed tumor vessels are tortuous, disorganized, highly permeable and dilated, causing irregular and inefficient perfusion. Angiogenesis results from an imbalance between angiogenic stimulators and inhibitors. In glioblastoma, a plethora of growth factors act as angiogenic stimulators, including basic fibroblast growth factor, angiopoietins, platelet-derived growth factor, IL-8 and hepatocyte growth factor (32). However, VEGFA is the major promoting stimulator of glioblastoma angiogenesis and is directly correlated with poor prognosis and the degree of malignancy (4). VEGFA is upregulated in, and mainly secreted by, pseudo-palisading glioma cells of necrotic areas. In a paracrine manner, it then activates VEGFRs on vascular endothelial cells to promote their activation, proliferation, migration and tube formation and to inhibit apoptosis (33,34). Therefore, targeting VEGFA or VEGFRs is a promising therapeutic approach for the treatment of glioblastoma. Bevacizumab, a humanized monoclonal anti-VEGFA antibody, has received approval from the United States Food and Drug Administration for the treatment of glioblastoma (35). Bevacizumab prolongs progression-free survival; however, there is no significant benefit to patient overall survival (35). Therefore, the identification of novel biomarkers and an

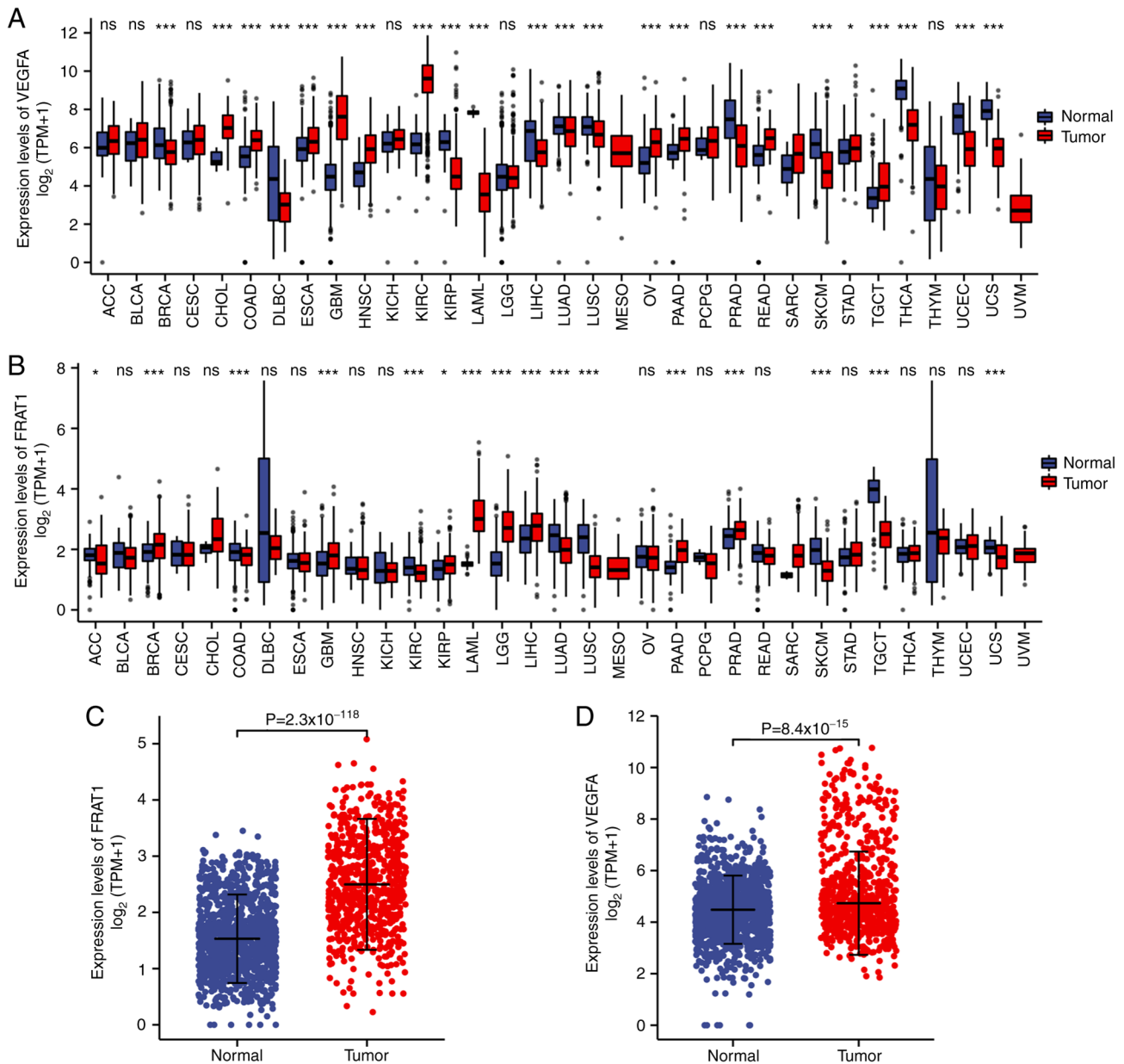


Figure 1. mRNA expression status of FRAT1 and VEGFA. (A and B) Wilcoxon rank sum test was used to analyze the differences in expression of FRAT1 and VEGFA in normal and tumor samples using the TCGA pan-cancer database. The expression levels of FRAT1 and VEGFA were demonstrated to be significantly higher in most tumor tissues compared with normal tissues, especially in GBM, although a small number of cancer tissues did display lower expression levels than in the normal tissues. (C and D) FRAT1 and VEGFA expression levels in normal and tumor samples of TCGA in GBMLGG. FRAT1, frequently rearranged in advanced T-cell lymphomas-1; TCGA, The Cancer Genome Atlas; GBMLGG, glioblastoma and low-grade glioma; ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large b-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma; ns, not significant. * $P < 0.05$ and ** $P < 0.001$.

increased understanding of VEGFA molecular signaling pathways are required to enhance the clinical efficacy of anti-VEGFA therapy.

The Wnt/ β -catenin signaling pathway is highly conserved in metazoan organisms and serves a critical role in embryonic development and carcinogenesis (36,37). As a positive regulator of Wnt/ β -catenin signaling, FRAT1 is highly upregulated in various human cancers, such as non-small

cell lung cancer (NSCLC) (17,38), chondrosarcoma (39), ovarian cancer (18), colon cancer (19), gastric cancer (12) and esophageal cancer (20). This upregulation commonly leads to high malignant potential, growth to the point of lethality, and subsequently, poor patient prognosis in these diseases. However, the role of FRAT1 in human gliomas remains to be elucidated. Our previous study indicated that FRAT1 expression levels are highly upregulated in human glioma tissues,

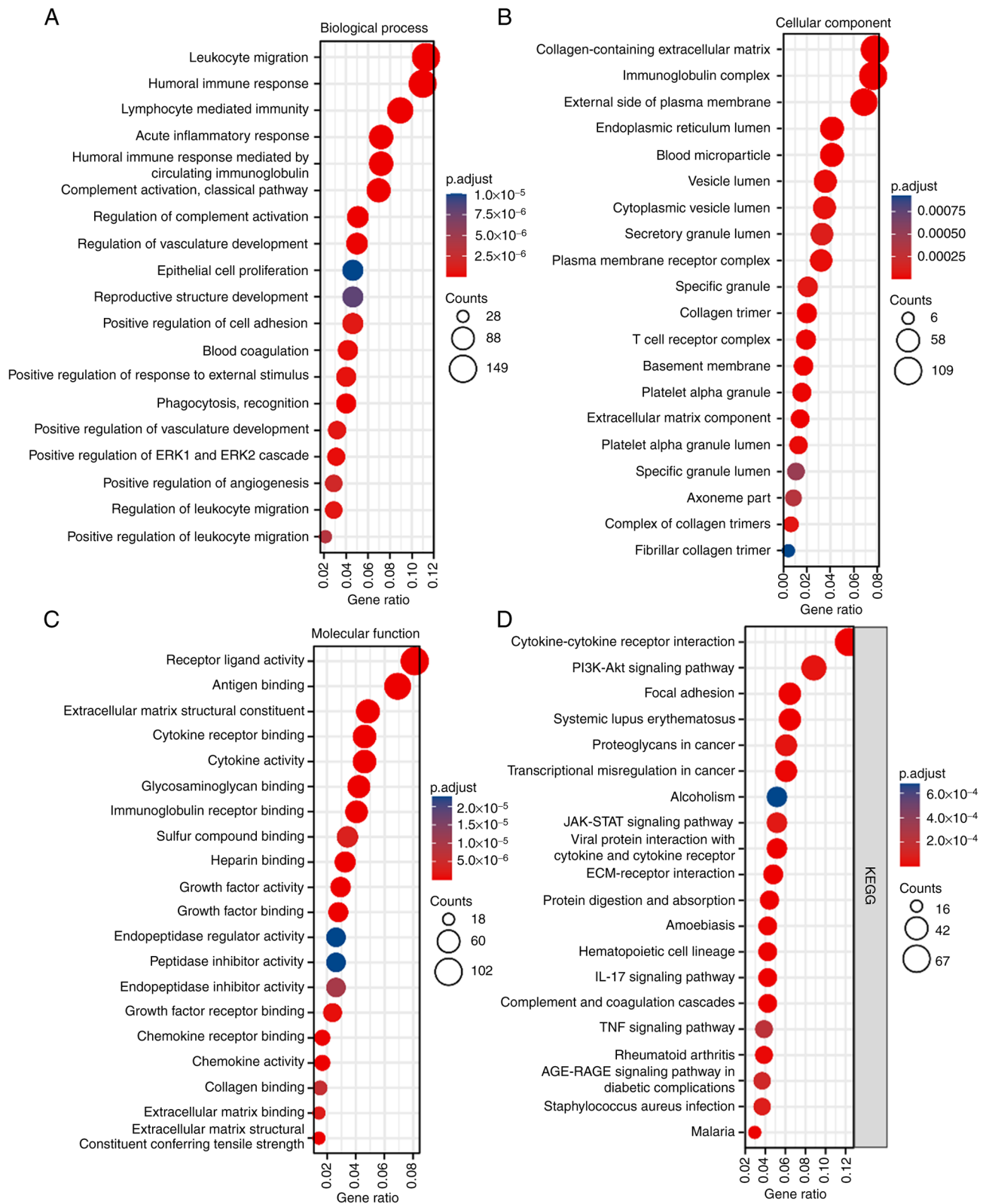


Figure 2. Functional enrichment of FRAT1 in glioblastoma and low-grade glioma. (A-C) Gene Ontology enrichment analysis of FRAT1. (D) KEGG enrichment analysis of FRAT1. FRAT1, frequently rearranged in advanced T-cell lymphomas-1; KEGG, Kyoto Encyclopedia of Genes and Genomes; ECM, extracellular matrix; AGE-RAGE, advanced glycation end products-receptor for advanced glycation end products; P.adj, adjusted P-value.

but low or absent in healthy brain tissues (40). Furthermore, upregulated FRAT1 expression is closely correlated with pathological grade, increased proliferation, invasiveness, reduced apoptosis and poor prognosis in patients with

glioma (21,41). Moreover, FRAT1 knockdown inhibits cellular proliferation, migration and invasiveness and results in G₀/G₁ cell cycle arrest in human glioblastoma U251 cells, as well as suppressing tumorigenesis in nude mice (23). It can

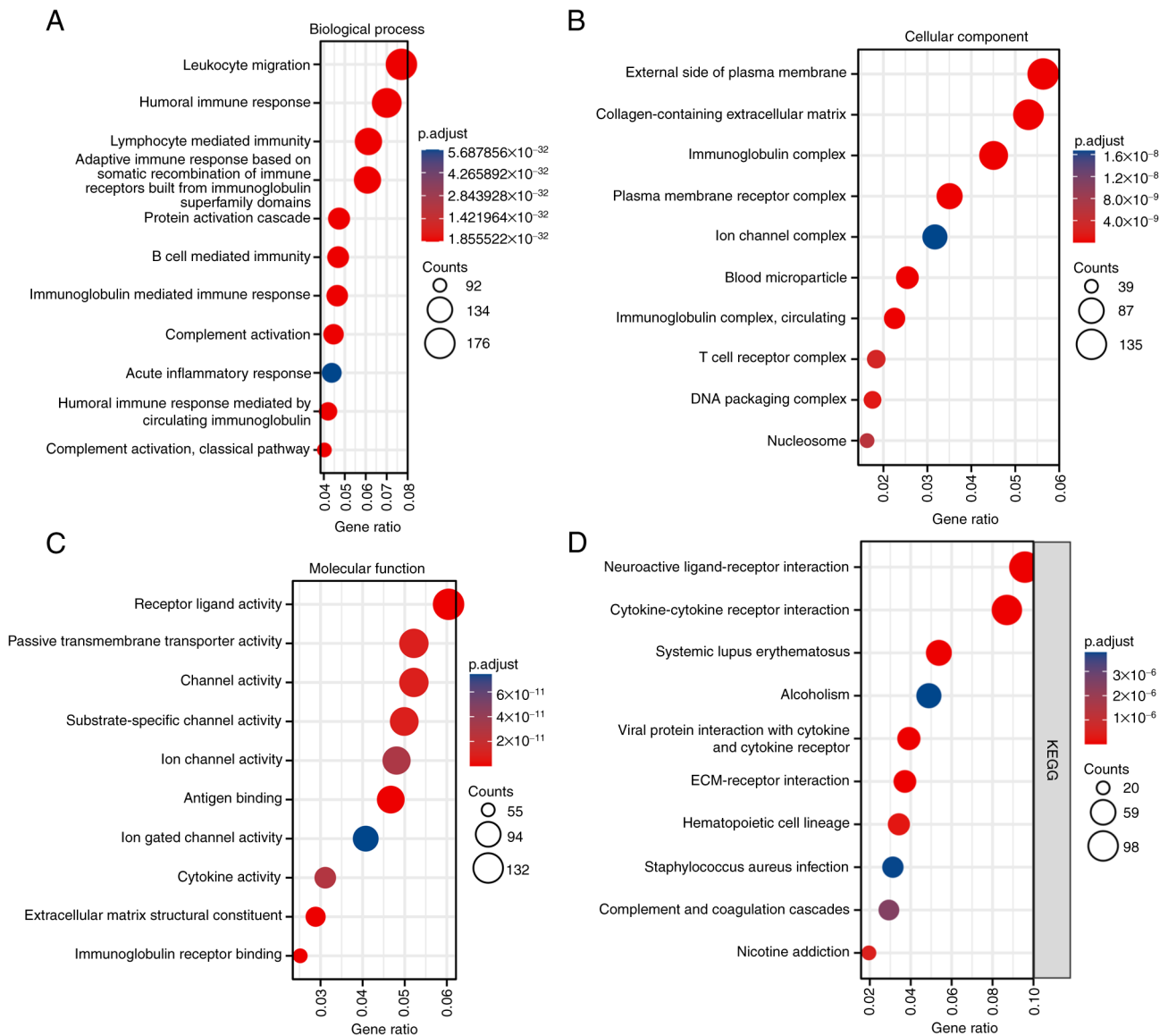


Figure 3. Functional enrichment of VEGFA in glioblastoma and low-grade glioma. (A-C) Gene Ontology enrichment analysis of VEGFA. (D) KEGG enrichment analysis of VEGFA. KEGG, Kyoto Encyclopedia of Genes and Genomes; ECM, extracellular matrix; P.adj, adjusted P-value.

therefore be hypothesized that FRAT1 acts as an oncogene in human gliomas.

In the Wnt signaling pathway, FRAT1 directly competes with axin for the same GSK-3 β binding sites, resulting in GSK-3 β inactivation and dissociation from the destruction complex, consisting of adenomatous polyposis coli (APC), axin, casein kinase I and GSK-3 β , thereby inhibiting GSK-3 β -mediated phosphorylation of β -catenin and its degradation (12,14,42,43). Subsequently, stabilized and dephosphorylated β -catenin accumulates in the nucleus, where it forms a transcriptional complex with T-cell factor (TCF)4/lymphoid-enhancing factor 1 to activate oncogenic target genes, such as cyclin D1 and c-myc (14,44,45). These aforementioned genes serve important roles in the development and formation of various tumors. Using immunohistochemistry, the upregulation of FRAT1 was previously found to be significantly correlated with the aberrant expression of β -catenin in human glioma tissues (21). Moreover, numerous studies have reported that the expression levels

of FRAT1 and β -catenin are positively associated in human gastric adenocarcinoma cells, NSCLC, ovarian cancer, colorectal cancer and esophageal cancer (18,20,44,46,47). Fan *et al* (48) also reported that knocking down FRAT1 inhibited β -catenin, cyclin D1 and c-myc expression by regulating the Wnt/ β -catenin signaling pathway in hepatocellular carcinoma cells. Low FRAT1 expression has also been demonstrated to reduce β -catenin mRNA and protein expression levels in a glioma stem cell xenograft mice model, where their expression levels were positively correlated (49). It can therefore be hypothesized that FRAT1 acts as an oncogene in human glioblastoma via activation of the Wnt/ β -catenin signaling pathway.

There is increasing evidence to support the significant role of the Wnt signaling pathway in angiogenesis in both physiological and pathological conditions, including cancers. This signaling pathway influences cellular proliferation, survival, differentiation, migration and apoptosis (8-11) and previous studies (50-52) have reported that certain Wnt antagonists

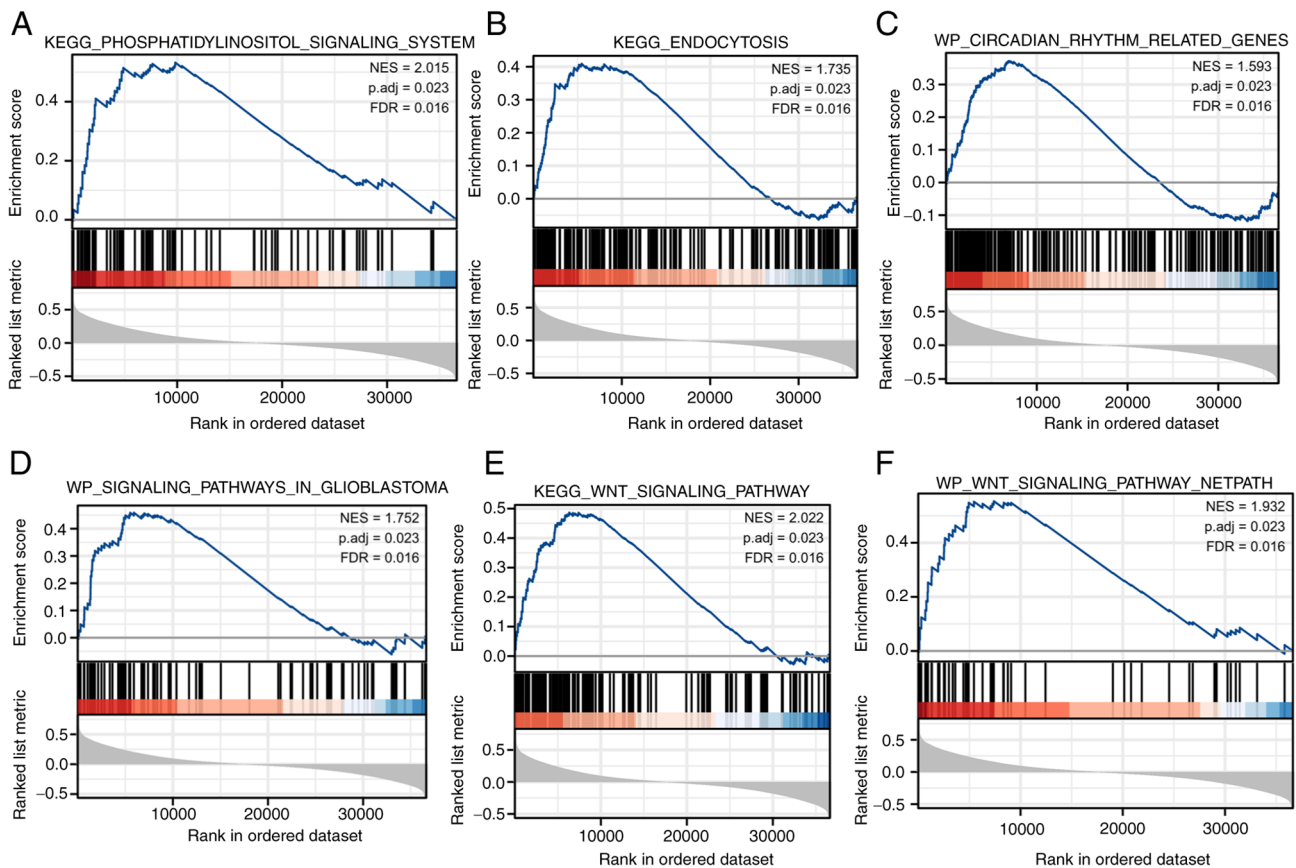


Figure 4. FRAT1 enrichment plot using Gene Set Enrichment Analysis. (A-F) Data on the left in red is significantly enriched (FRAT1 high expression group). NES, normalized enrichment score; P.adj, adjusted P-value; FDR, false discovery rate; KEGG, Kyoto Encyclopedia of Genes and Genomes; WP, WikiPathways.

downregulate VEGFA expression. For example, in hepatocellular carcinoma, blocking the Wnt/ β -catenin signaling pathway using Wnt inhibitory factor 1 (WIF-1) decreased microvessel density (MVD) and VEGFA secretion. WIF-1 also inhibited the tube formation and migration of human microvascular endothelial and mouse endothelial progenitor cells (50). Dickkopf-related protein 1, a Wnt antagonist, has been demonstrated to markedly reduce the expression levels of VEGFA and β -catenin in the retinal pigment epithelium of very low-density lipoprotein receptor (Vldlr)^{-/-} mice, as well as in HUVECs transfected with Vldlr siRNAs (51). R-spondin3 (Rspo3) is a potent activator of the Wnt/ β -catenin signaling pathway. Rspo3 has been reported to promote VEGFA expression and *in vitro* angiogenesis via activation of the Wnt/ β -catenin signaling pathway, whereas disrupting Rspo3 was demonstrated to induce vascular defects in the placenta and yolk sac (52).

Moreover, components of the Wnt/ β -catenin signaling pathway also regulate VEGFA secretion. β -catenin is the central regulator of the Wnt/ β -catenin pathway and ectopic activation of β -catenin has been shown to upregulate VEGFA expression in colon cancer (53,54). The VEGFA gene promoter contains seven consensus binding sites for β -catenin/TCF and functional deletion of APC increases VEGFA mRNA and protein expression by activating β -catenin (54). It can therefore be hypothesized that VEGFA is a direct target of the β -catenin gene. Frizzled is a membrane receptor for Wnt ligands. VEGFA mRNA expression is significantly reduced in the ovarian

cells of Fzd4^{-/-} mice, whereas apoptosis is increased (55). Furthermore, Wnt1 and VEGFA protein expression levels are significantly upregulated in atherosclerotic rats and are positively correlated (56). Moreover, the Wnt target gene c-myc is a potent inducer of angiogenesis during embryonic development and tumorigenesis, and in association with hypoxia-inducible factor-1 (HIF-1), dysregulated c-myc promotes VEGFA expression and angiogenesis (57). GSK-3 β downregulates β -catenin to inhibit differentiation, migration, VEGFA expression and angiogenesis in HUVECs (58). Guo *et al* (59) reported that lithium increases the levels of GSK-3 β phosphorylation and promotes VEGFA secretion in a dose-dependent manner, in both human brain microvascular endothelial cells and primary rat cortical astrocytes *in vitro*. Zhao *et al* (60) demonstrated that the overexpression of GSK-3 β not only suppresses the expression of HIF-1 α , β -catenin and VEGFA, but also inhibits proliferation and angiogenesis in human glioma cells. Furthermore, by regulating HIF-1 α expression under normoxic conditions, aberrant activation of the Wnt/ β -catenin signaling pathway induces various other signaling cascades to promote VEGFA expression, including the EGFR/PI3K/Akt and STAT3 signaling pathways (61). Collectively, these findings suggest that VEGFA may be a promising means of targeting the Wnt/ β -catenin signaling cascade in angiogenesis.

Our previous study demonstrated that the degree of MVD was significantly associated with FRAT1 expression in human glioma tissues (21), indicating that FRAT1 may be a positive angiogenic regulator in human glioma.

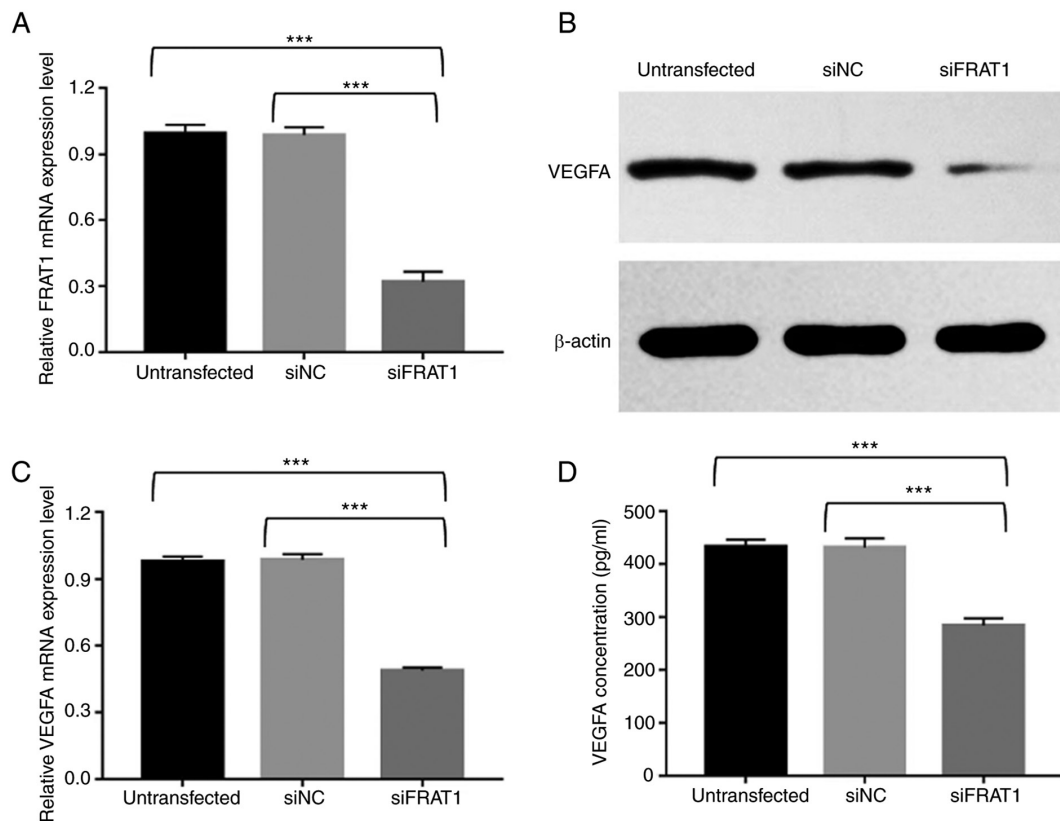


Figure 5. Expression levels of FRAT1 and VEGFA in untransfected, siNC and siFRAT1 U251 cells. (A) FRAT1 mRNA expression levels in siFRAT1 U251 cells were significantly decreased and there was no significant difference between the untransfected and siNC groups. Subsequently, western blotting, reverse transcription-quantitative PCR and ELISA were used to determine VEGFA levels in untransfected, siNC and siFRAT1 U251 cells. (B) VEGFA protein expression levels were markedly reduced in the siFRAT1 group compared with the untransfected and siNC groups. No significant difference was seen between the untransfected and siNC groups. (C) VEGFA mRNA expression levels were significantly decreased in the siFRAT1 group compared with the untransfected and siNC groups. (D) VEGFA concentration in the supernatants of siFRAT1 cells were significantly lower compared with the transfected and siNC groups. *** $P < 0.001$. FRAT1, frequently rearranged in advanced T-cell lymphomas-1; si, small interfering RNA; NC, negative control.

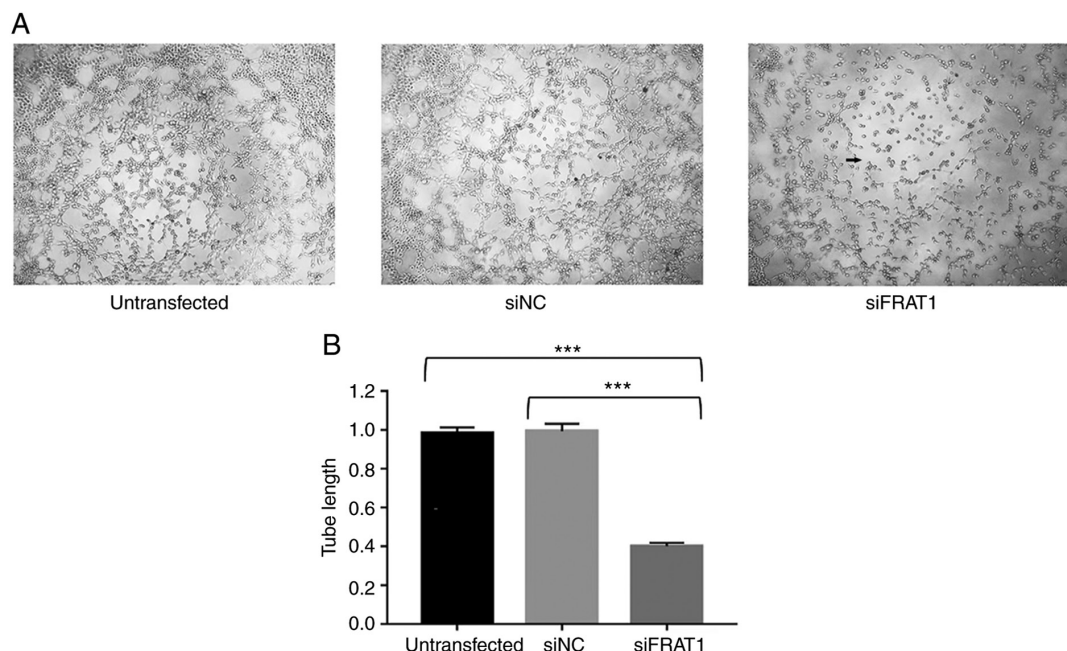


Figure 6. Effects of untransfected, siNC and siFRAT1 U251 cell supernatants on HUVEC angiogenesis. HUVECs were cultured at 37°C for 6 h with the supernatants collected from untransfected, siNC and siFRAT1 U251 cells, after which tube formation capacity was assessed. (A) HUVEC angiogenic ability was markedly impaired in the siFRAT1 group (black arrow), compared with the untransfected and siNC groups. Magnification, x40. (B) Relative changes in tube length in the siFRAT1 group were significantly decreased compared with those of the untransfected and siNC groups. HUVEC angiogenic ability or tube length in the untransfected and siNC groups demonstrated no significant difference. *** $P < 0.001$. si, small interfering (RNA); NC, negative control; FRAT1, frequently rearranged in advanced T-cell lymphomas-1; ns, not significant.

With the development of sequencing technology and the production of large amounts of sequencing data, tumor gene expression data analysis was enabled. In the present study, gene expression analysis in the tumor was performed, which specifically explored the role of genes in the tumor, from which valuable biomarkers for diagnosis could be determined. To the best of our knowledge, the role of FRAT1 and VEGFA in glioma, prognosis and diagnosis, was investigated for the first time in the present study, based on using the TCGA database and physiological and biochemical methods. The results demonstrated that the expression of FRAT1 and VEGFA in pan-cancer was similar to that seen in certain previous studies (62,63). Furthermore, FRAT1 and VEGFA mRNA expression levels were significantly increased in GBMLGG compared with normal tissues. These results indicated that FRAT1 and VEGFA are highly associated with the occurrence of GBMLGG.

Key terms identified presented in the differentially expressed genes in FRAT1 and VEGFA GO enrichment analysis, such as 'blood microparticle', 'immunoglobulin complex', 'extracellular matrix structural constituent', indicated that FRAT1 may be related to the process of blood vessel formation. GSEA analysis of FRAT1 single gene correlation further explored the signaling pathway involved with FRAT1, further supporting the relationship between angiogenesis and tumorigenesis.

The aforementioned results indicated that FRAT1 may be a positive angiogenic regulator in human glioma. To confirm this hypothesis, the effects of FRAT1 knockdown on VEGFA expression and angiogenesis were analyzed using human U251 glioblastoma cells. The results demonstrated that FRAT1 knockdown significantly reduced the mRNA and protein expression levels of VEGFA and decreased VEGFA concentration in the cell supernatant. FRAT1 knockdown was also found to markedly inhibit HUVEC angiogenesis, which confirmed the results of our previous study. These results therefore indicated that FRAT1 may therefore be an important mediator for glioma angiogenesis that exerts its effects via VEGFA regulation.

In conclusion, the results of the present study indicated that FRAT1 may serve an important role in the regulation of angiogenesis via VEGFA regulation and that suppressing FRAT1 may inhibit angiogenesis in human glioblastoma U251 cells. Furthermore, this process may also involve the Wnt/ β -catenin signaling pathway. However, the primary limitation of the present study is the lack of additional cell lines, including glioblastoma U87 cells, glioma SHG44 cells and normal human brain cells to determine the effect of FRAT1 on angiogenesis in glioblastoma. Despite this limitation, the current findings have provided insights into the role of FRAT1, which appears to be a novel and valuable biomarker for the diagnosis and prognosis of human glioblastoma. Moreover, inhibiting angiogenesis by targeting FRAT1 may be a promising option for treating human glioblastoma. However, further investigation is required to elucidate the association between FRAT1 and angiogenesis in glioblastoma.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 81201991), the China Postdoctoral Science Foundation (grant no. 2015M571068 and 2016T90115) and the Beijing Postdoctoral Research Foundation (grant no. 2015ZZ-56 and 2016ZZ-43).

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BY, YQR, DL and GG contributed to the conception of the study and contributed significantly towards data analysis and manuscript preparation. BY, JPZ and GG performed the experiments. YQS, XGW, YQW, SLW and SHG analyzed the bioinformatics data, and participated in the conception and experimental design of the project. BY and GG confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The use of primary HUVECs was approved by the ethics committee of The First Hospital of Shanxi Medical University [approval no. (2021) Y10].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Ostrom QT, Cioffi G, Gittleman H, Patil N, Waite K, Kruchko C and Barnholtz-Sloan JS: CBTRUS statistical report: Primary brain and other central nervous system tumors diagnosed in the United States in 2012-2016. *Neuro Oncol* 21 (Suppl 5): v1-v100, 2019.
- Stupp R, Taillibert S, Kanner A, Read W, Steinberg D, Lhermitte B, Toms S, Idubai A, Ahluwalia MS, Fink K, *et al*: Effect of tumor-treating fields plus maintenance temozolomide vs maintenance temozolomide alone on survival in patients with glioblastoma: A randomized clinical trial. *JAMA* 318: 2306-2316, 2017.
- Kargiotis O, Rao JS and Kyritsis AP: Mechanisms of angiogenesis in gliomas. *J Neurooncol* 78: 281-293, 2006.
- Norden AD, Drappatz J and Wen PY: Antiangiogenic therapies for high-grade glioma. *Nat Rev Neurol* 5: 610-620, 2009.
- Ferrara N: VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer* 2: 795-803, 2002.
- Plate KH, Scholz A and Dumont DJ: Tumor angiogenesis and anti-angiogenic therapy in malignant gliomas revisited. *Acta Neuropathol* 124: 763-775, 2012.
- Hundsberger T, Reardon DA and Wen PY: Angiogenesis inhibitors in tackling recurrent glioblastoma. *Expert Rev Anticancer Ther* 17: 507-515, 2017.
- Masckauchan TN and Kitajewski J: Wnt/Frizzled signaling in the vasculature: New angiogenic factors in sight. *Physiology (Bethesda)* 21: 181-188, 2006.

9. Zhang B and Ma JX: Wnt pathway antagonists and angiogenesis. *Protein Cell* 1: 898-906, 2010.
10. Zerlin M, Julius MA and Kitajewski J: Wnt/Frizzled signaling in angiogenesis. *Angiogenesis* 11: 63-69, 2008.
11. Parmalee NL and Kitajewski J: Wnt signaling in angiogenesis. *Curr Drug Targets* 9: 558-564, 2008.
12. Saitoh T and Katoh M: FRAT1 and FRAT2, clustered in human chromosome 10q24.1 region, are up-regulated in gastric cancer. *Int J Oncol* 19: 311-315, 2001.
13. Jonkers J, Korswagen HC, Acton D, Breuer M and Berns A: Activation of a novel proto-oncogene, *Frat1*, contributes to progression of mouse T-cell lymphomas. *EMBO J* 16: 441-450, 1997.
14. Hagen T, Cross DA, Culbert AA, West A, Frame S, Morrice N and Reith AD: FRAT1, a substrate-specific regulator of glycogen synthase kinase-3 activity, is a cellular substrate of protein kinase A. *J Biol Chem* 281: 35021-35029, 2006.
15. Ferkey DM and Kimelman D: Glycogen synthase kinase-3 beta mutagenesis identifies a common binding domain for GBP and Axin. *J Biol Chem* 277: 16147-16152, 2002.
16. Yost C, Farr GH III, Pierce SB, Ferkey DM, Chen MM and Kimelman D: GBP, an inhibitor of GSK-3, is implicated in *Xenopus* development and oncogenesis. *Cell* 93: 1031-1041, 1998.
17. Zhang Y, Yu JH, Lin XY, Miao Y, Han Y, Fan CF, Dong XJ, Dai SD and Wang EH: Overexpression of *Frat1* correlates with malignant phenotype and advanced stage in human non-small cell lung cancer. *Virchows Arch* 459: 255-263, 2011.
18. Wang Y, Hewitt SM, Liu S, Zhou X, Zhu H, Zhou C, Zhang G, Quan L, Bai J and Xu N: Tissue microarray analysis of human FRAT1 expression and its correlation with the subcellular localisation of beta-catenin in ovarian tumours. *Br J Cancer* 94: 686-691, 2006.
19. Zhu K, Guo J, Wang H and Yu W: FRAT1 expression regulates proliferation in colon cancer cells. *Oncol Lett* 12: 4761-4766, 2016.
20. Wang Y, Liu S, Zhu H, Zhang W, Zhang G, Zhou X, Zhou C, Quan L, Bai J, Xue L, *et al*: FRAT1 overexpression leads to aberrant activation of beta-catenin/TCF pathway in esophageal squamous cell carcinoma. *Int J Cancer* 123: 561-568, 2008.
21. Guo G, Zhong CL, Liu Y, Mao XG, Zhang Z, Jin J, Liu J, Yang L, Mao JM, Guo YH and Zhao YL: Overexpression of FRAT1 is associated with malignant phenotype and poor prognosis in human gliomas. *Dis Markers* 2015: 289750, 2015.
22. Chandrashekar DS, Babel B, Balasubramanya SAH, Creighton CJ, Ponce-Rodriguez I, Chakravarthi BVSK and Varambally S: UALCAN: A portal for facilitating tumor subgroup gene expression and survival analyses. *Neoplasia* 19: 649-658, 2017.
23. Hanzelmann S, Castelo R and Guinney J: GSVA: Gene set variation analysis for microarray and RNA-Seq data. *BMC Bioinformatics* 14: 7, 2013.
24. Ding J, Liang Z, Feng W, Cai Q and Zhang Z: Integrated bioinformatics analysis reveals potential pathway biomarkers and their interactions for clubfoot. *Med Sci Monit* 26: e925249, 2020.
25. Yu G, Wang LG, Han Y and He QY: clusterProfiler: An R package for comparing biological themes among gene clusters. *OMICS* 16: 284-287, 2012.
26. Subramanian A, Kuehn H, Gould J, Tamayo P and Mesirov JP: GSEA-P: A desktop application for gene set enrichment analysis. *Bioinformatics* 23: 3251-3253, 2007.
27. Guo G, Kuai D, Cai S, Xue N, Liu Y, Hao J, Fan Y, Jin J, Mao X, Liu B, *et al*: Knockdown of FRAT1 expression by RNA interference inhibits human glioblastoma cell growth, migration and invasion. *PLoS One* 8: e61206, 2013.
28. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
29. Robinson RL, Sharma A, Bai S, Heneidi S, Lee TJ, Kodeboyina SK, Patel N and Sharma S: Comparative STAT3-regulated gene expression profile in renal cell carcinoma subtypes. *Front Oncol* 9: 72, 2019.
30. Onishi M, Ichikawa T, Kurozumi K and Date I: Angiogenesis and invasion in glioma. *Brain Tumor Pathol* 28: 13-24, 2011.
31. Cea V, Sala C and Verpelli C: Antiangiogenic therapy for glioma. *J Signal Transduct* 2012: 483040, 2012.
32. Folkman J: What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 82: 4-6, 1990.
33. Ferrara N, Gerber HP and LeCouter J: The biology of VEGF and its receptors. *Nat Med* 9: 669-676, 2003.
34. Chi AS, Sorensen AG, Jain RK and Batchelor TT: Angiogenesis as a therapeutic target in malignant gliomas. *Oncologist* 14: 621-636, 2009.
35. Irizarry LR, Hambardzumyan D, Nakano I, Gladson CL and Ahluwalia MS: Therapeutic targeting of VEGF in the treatment of glioblastoma. *Expert Opin Ther Targets* 16: 973-984, 2012.
36. Macdonald BT, Tamai K and He X: Wnt/beta-catenin signaling: Components, mechanisms, and diseases. *Dev Cell* 17: 9-26, 2009.
37. Clevers H and Nusse R: Wnt/beta-catenin signaling and disease. *Cell* 149: 1192-1205, 2012.
38. Zhang Y, Han Y, Zheng R, Yu JH, Miao Y, Wang L and Wang EH: Expression of *Frat1* correlates with expression of beta-catenin and is associated with a poor clinical outcome in human SCC and AC. *Tumour Biol* 33: 1437-1444, 2012.
39. He L, Yang Z, Zhou J and Wang W: The clinical pathological significance of FRAT1 and ROR2 expression in cartilage tumors. *Clin Transl Oncol* 17: 438-445, 2015.
40. Guo G, Mao X, Wang P, Liu B, Zhang X, Jiang X, Zhong C, Huo J, Jin J and Zhuo Y: The expression profile of FRAT1 in human gliomas. *Brain Res* 1320: 152-158, 2010.
41. Guo G, Liu B, Zhong C, Zhang X, Mao X, Wang P, Jiang X, Huo J, Jin J, Liu X and Chen X: FRAT1 expression and its correlation with pathologic grade, proliferation, and apoptosis in human astrocytomas. *Med Oncol* 28: 1-6, 2011.
42. Nager M, Bhardwaj D, Cantí C, Medina L, Nogués P and Herreros J: beta-catenin signalling in glioblastoma multiforme and glioma-initiating cells. *Chemother Res Pract* 2012: 192362, 2012.
43. Dajani R, Fraser E, Roe SM, Yeo M, Good VM, Thompson V, Dale TC and Pearl LH: Structural basis for recruitment of glycogen synthase kinase 3beta to the axin-APC scaffold complex. *EMBO J* 22: 494-501, 2003.
44. Jamieson C, Sharma M and Henderson BR: Wnt signaling from membrane to nucleus: beta-catenin caught in a loop. *Int J Biochem Cell Biol* 44: 847-850, 2012.
45. Sareddy GR, Panigrahi M, Challa S, Mahadevan A and Babu PP: Activation of Wnt/beta-catenin/TCF signaling pathway in human astrocytomas. *Neurochem Int* 55: 307-317, 2009.
46. Yu Q, Shang LU, Yu H, Yang Z and Xu D: Silencing of FRAT1 by siRNA inhibits the proliferation of SGC7901 human gastric adenocarcinoma cells. *Biomed Rep* 4: 223-226, 2016.
47. Zheng K, Zhou X, Yu J, Li Q, Wang H, Li M, Shao Z, Zhang F, Luo Y, Shen Z, *et al*: Epigenetic silencing of miR-490-3p promotes development of an aggressive colorectal cancer phenotype through activation of the Wnt/beta-catenin signaling pathway. *Cancer Lett* 376: 178-187, 2016.
48. Fan WH, Du FJ, Liu XJ and Chen N: Knockdown of FRAT1 inhibits hypoxia-induced epithelial-to-mesenchymal transition via suppression of the Wnt/beta-catenin pathway in hepatocellular carcinoma cells. *Oncol Rep* 36: 2999-3004, 2016.
49. Guo G, Liu J, Ren Y, Mao X, Hao Y, Zhong C, Chen X, Wang X, Wu Y, Lian S, *et al*: FRAT1 enhances the proliferation and tumorigenesis of CD133(+)Nestin(+) glioma stem cells in vitro and in vivo. *J Cancer* 11: 2421-2430, 2020.
50. Hu J, Dong A, Fernandez-Ruiz V, Shan J, Kawa M, Martínez-Ansó E, Prieto J and Qian C: Blockade of Wnt signaling inhibits angiogenesis and tumor growth in hepatocellular carcinoma. *Cancer Res* 69: 6951-6959, 2009.
51. Chen Y, Hu Y, Lu K, Flannery JG and Ma JX: Very low-density lipoprotein receptor, a negative regulator of the wnt signaling pathway and choroidal neovascularization. *J Biol Chem* 282: 34420-34428, 2007.
52. Kazanskaya O, Ohkawara B, Herault M, Wu W, Maltry N, Augustin HG and Niehrs C: The Wnt signaling regulator R-spondin 3 promotes angioblast and vascular development. *Development* 135: 3655-3664, 2008.
53. Zhang X, Gaspard JP and Chung DC: Regulation of vascular endothelial growth factor by the Wnt and K-ras pathways in colonic neoplasia. *Cancer Res* 61: 6050-6054, 2001.
54. Easwaran V, Lee SH, Inge L, Guo L, Goldbeck C, Garrett E, Wiesmann M, Garcia PD, Fuller JH, Chan V, *et al*: beta-Catenin regulates vascular endothelial growth factor expression in colon cancer. *Cancer Res* 63: 3145-3153, 2003.
55. Hsieh M, Boerboom D, Shimada M, Lo Y, Parlow AF, Luhmann UFO, Berger W and Richards JS: Mice null for frizzled4 (*Fzd4*^{-/-}) are infertile and exhibit impaired corpora lutea formation and function. *Biol Reprod* 73: 1135-1146, 2005.
56. Du J and Li J: The role of wnt signaling pathway in atherosclerosis and its relationship with angiogenesis. *Exp Ther Med* 16: 1975-1981, 2018.

57. Kim JW, Gao P, Liu YC, Semenza GL and Dang CV: Hypoxia-inducible factor 1 and dysregulated c-Myc cooperatively induce vascular endothelial growth factor and metabolic switches hexokinase 2 and pyruvate dehydrogenase kinase 1. *Mol Cell Biol* 27: 7381-7393, 2007.
58. Skurk C, Maatz H, Rocnik E, Bialik A, Force T and Walsh K: Glycogen-Synthase Kinase3beta/beta-catenin axis promotes angiogenesis through activation of vascular endothelial growth factor signaling in endothelial cells. *Circ Res* 96: 308-318, 2005.
59. Guo S, Arai K, Stins MF, Chuang DM and Lo EH: Lithium upregulates vascular endothelial growth factor in brain endothelial cells and astrocytes. *Stroke* 40: 652-655, 2009.
60. Zhao P, Li Q, Shi Z, Li C, Wang L, Liu X, Jiang C, Qian X, You Y, Liu N, *et al*: GSK-3beta regulates tumor growth and angiogenesis in human glioma cells. *Oncotarget* 6: 31901-31915, 2015.
61. Vallée A, Guillemin R and Vallée JN: Vasculogenesis and angiogenesis initiation under normoxic conditions through Wnt/ β -catenin pathway in gliomas. *Rev Neurosci* 29: 71-91, 2018.
62. Xiao SF, Tang HR, Bai Y, Zou RC, Ren ZF, Wu XS, Shi ZT, Lan S, Liu W, Wu TG, *et al*: Swertiamarin suppresses proliferation, migration, and invasion of hepatocellular carcinoma cells via negative regulation of FRAT1. *Eur J Histochem* 64: 271-278, 2020.
63. Tian W, Lei N, Guo R, Yuan Z and Chang L: Long non-coding RNA DANCER promotes cervical cancer growth via activation of the Wnt/ β -catenin signaling pathway. *Cancer Cell Int* 20: 61, 2020.