miR-24 regulates angiogenesis in gliomas

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Abstract. Gliomas are one of the most common and most aggressive types of central nervous system tumor. Angiogenesis is an important basis for the growth of solid tumors, including gliomas, which is regulated by microRNAs (miRNAs). However, the mechanism remains unclear. Recently, it was demonstrated that miR-24 was upregulated in gliomas, so the aim of the present study is to establish whether the dysregulation of miR-24 in glioma cells promotes microvascular proliferation of endothelial cells (ECs), and to investigate the potential mechanism. miR-24 was overexpressed or downregulated in U251 glioma cell line cells using miR-24 mimics or inhibitors, respectively. Subsequently, the effects of conditional medium from miR-24 mimic- or inhibitor-transfected U251 cells on cell viability, migration and angiogenesis of human umbilical vein ECs (HUVECs) were examined. The expression levels of vascular endothelial growth factor (VEGF) mRNA, basic fibroblast growth factor (bFGF) mRNA, epidermal growth factor (EGF) mRNA, transforming growth factor (TGF)-β mRNA, matrix metalloproteinase (MMP)-2 mRNA and MMP-9 mRNA, and the mRNA and protein levels of VEGF and TGF-β in miR-24 mimic- or inhibitor-transfected U251 cells were obtained by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. The effects of conditional medium from miR-24 mimic- or inhibitor-transfected U251 cells on expression levels of VEGF mRNA, TGF-β mRNA, MMP-2 mRNA and MMP-9 mRNA, and mRNA and protein expression levels of VEGF and TGF-β, and intracellular AKT and β-catenin signaling in HUVECs were also examined. The results indicated that the conditional medium from miR-24 mimic-transfected U251 cells exhibited significantly increased cell viability, cell migration and tube formation of HUVECs. By contrast, the conditional medium from miR-24 inhibitor-transfected U251 cells exhibited significantly decreased cell viability, cell migration and tube formation of HUVECs. Enforced expression of miR-24 in U251 cells may promote the cell viability and angiogenesis of HUVECs. The mRNA expression levels of VEGF, bFGF, EGF, TGF-β, MMP-2 and MMP-9 in U251 cells were significantly increased by miR-24 mimics. Western blot detection confirmed the increased levels of VEGF and TGF-β protein expression in U251 by miR-24 mimics, and the decrease of VEGF and TGF-β protein expression levels in U251 by miR-24 inhibitors. The conditional medium from miR-24 mimic-transfected U251 cells increased the expression levels of the angiogenesis-associated factors, including VEGF, TGF-β, MMP-2, and MMP-9. By contrast, reduced expression of miR-24 in U251 cells may downregulate the expression of those angiogenesis-associated factors. Thus, miR-24 in U251 cells may be important in the angiogenesis of HUVECs via VEGF and TGF-β, and the intracellular signaling of AKT and β-catenin may be involved in this process.

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Introduction

Gliomas are one of the most common and most aggressive types of central nervous system tumor. Gliomas account for approximately 35-60% of all primary intracranial tumors (1). Gliomas are derived from various types of glial cell tumor, which are divided into four categories, including astrocytoma, medulloblastoma, oligodendroglioma and ependymoma. Among these, glioblastomas are the most common, accounting for ~55% of all intracranial neoplasms. According to the pathological grading defined by the World Health Organization in 2007, gliomas are divided into grade I, II, III and IV (2). Glioblastoma multiforme (GBM) are grade IV. The clinical features of glioma include rapid growth, high infiltration, and with no obvious boundary between tumor tissue and normal brain tissue. It is difficult to achieve complete removal of tumor tissues via surgery; thus, the recurrence rate is high. In addition, due to the existence of the blood-brain barrier, antineoplastic drugs do not readily enter the brain. Although

chemotherapy combined with radiotherapy is often used to treat glioma, the outcome remains unsatisfactory. Previous evidence indicates that the median survival time in patients with newly diagnosed GBM is only 14.6 months despite the use of radiotherapy, chemotherapy and surgical intervention (3). A large number of studies have shown that gliomas remain one of the worst types of tumor with poor prognosis (4,5). Therefore, it is considered to be of great importance to elucidate the underlying mechanism of the occurrence and development of gliomas, and develop more effective targets to improve therapeutic strategies against gliomas.

Angiogenesis is an important basis for the growth of solid tumors, including gliomas (6,7). The rapid growth, invasion, metastasis and tumor recurrence of gliomas are closely associated with angiogenesis. Studies have demonstrated that glioma vascular endothelial hyperplasia increased significantly with glioma malignancy, which provides strong evidence that angiogenesis is an important feature of the development of glioma (8,9). Vascular endothelial growth factor (VEGF) is important in glioma angiogenesis (10). The use of anti-VEGF antibody in rat models of glioma *in vivo* demonstrated that angiogenesis was significantly inhibited, while there no inhibitory effect was observed in glioma cells *in vitro* (11,12). Thus, VEGF promotes the proliferation of ECs, and angiogenesis in glioma. Currently, microvascular proliferation is considered to be one of the most important criteria for malignant grading of glioma.

Gliomas exhibit a strong invasive ability (13). Tumor angiogenesis is vital in tumor invasion. In recent years, with the discovery of microRNAs (miRNA) and their function in in-depth investigations, it was identified that miRNA contributes to the regulation of angiogenesis in glioma formation, thus affecting the invasion ability of gliomas (14,15). Previous studies demonstrated that miR-24 was upregulated in glioblastoma tissue (16-18) and promotes cell proliferation in glioma cells via cooperative regulation of MAX interactor 1, dimerization protein (19). Furthermore, miR-24 regulates the proliferation and invasion of gliomas through suppression of tumorigenicity 7 like via β-catenin/transcription factor 4 signaling (20). The migration of human umbilical vein endothelial cells (HUVECs) is an important process (21), and HUVECs are a major contributor of angiogenesis (22,23). The aim of the present study was to investigate whether the dysregulation of miR-24 in glioma cells promotes microvascular proliferation of ECs, and to investigate the possible underlying mechanism.

Materials and methods

Cell culture and transfection. The human U251 glioma cell line and HUVECs were purchased from Shanghai Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) containing Gibco 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2.

The miR-24 mimics and inhibitor were chemically synthesized and purified by high-performance liquid choromatography (Shanghai GenePharma Co., Ltd., Shanghai, China). The primer sequences were as follows: Forward, 5'-UUCUCC GAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACAC GUUCGGAGAATT-3' for the negative control (NC); CUG UUCCUGCUGAACUGAGCCA for the miR-24 inhibitor; forward, 5'-UGGCUCAGUUCAGCAGGAACAG-3' and reverse, 5'-GUUCCUGCUGAACUGAGCCAUU-3' for the miR-24-3p mimic. Cell transfection was performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. For 48 h post-transfection, the changes of target genes were confirmed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The expression levels of miR-24 in the culture medium were detected by RT-qPCR. The levels of VEGFA was detected using an ELISA kit (cat. no. RS10115B; SHRQSW, Shanghai, China; http://www.shrqsw.com/) according to the manufacturer's instructions.

RNA extraction and RT-qPCR. Total RNA was extracted from cells that were transfected with mimic NC, mimic, inhibitor NC or inhibitor using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and were reverse-transcribed using a miR-Cute miRNA First Strand cDNA kit (KR201-02; TianGen Biotech Co., Ltd., Beijing, China). The sequences of the primers for mir-24 were as follows: Forward, 5'-UUC UCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGA CACGUUCGGAGAATT-3' forhsa-miR-24-3p; and forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'AACGCT TCACGAATTTGCGT-3' for U6. The RNA was quantified and checked for purity by spectrophotometry. The expression level of miR-24-3p was quantitated using a miRcute miRNA qPCR detection kit (FP401; TianGen Biotech Co., Ltd.) and U6 RNA served as an internal standard. The thermocycling conditions were as follows: 94˚C for 2 min, followed by 40 cycles of 94˚C for 20 sec and 60˚C for 34 sec. The comparative Cq ($\Delta\Delta$Cq) method (24) was used to determine the expression fold change.

Western blot analysis. Cells were lysed in radioimmunoprecipitation assay lysis buffer (BioTeke Corporation, Beijing, China) and concentration was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Proteins (30 μg per lane) were loaded for 10% SDS-PAGE and transferred to an Immobilon-P membrane (EMD Millipore, Billerica, MA, USA). The membrane was incubated with the following primary antibodies: Anti-VEGF (cat. no. ab46154; 1:1,000; Abcam, Cambridge, USA), anti-tumor growth factor (TGF)-β1 (cat. no. ab92486; 1:1,000; Abcam), anti-β-catenin (cat. no. ab32572; 1:1,000; Abcam), anti-AKT (cat. no. 4691s; 1:2,000; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-pAKT (cat. no. 4060s; 1:2,000; Cell Signaling Technology, Inc.) and anti-GAPDH (cat. no. ab181602; 1:5,000; Abcam) overnight at 4˚C, and incubated for 1 h with a horseradish Peroxidase-conjugated goat anti-rabbit (cat. no. 65-6120; 1:3,000; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature. The immunoblot was detected by Enhanced chemiluminescence Supersignal® western blotting detection reagents (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol using ChemiDox XRS + (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and densitometry was analyzed by Gelpro analyze 4.2 (Media Cybernetics, Inc., Rockville, MD, USA).

Cell viability. Cells ($2x10^3$ cells/well) were cultured in 96-well plates at 24, 48 and 72 h post-transfection. MTT reagent (20 μl; 5 mg/ml) was added directly to the medium and incubated at 37˚C, in a 5% CO_2 incubator for 4 h. After removing the supernatants, 150 μl dimethyl sulfoxide was added to dissolve the formazan crystals and mixed thoroughly for 10 min. Optical densities (ODs) at a wavelength of 570 nm were measured using the culture medium as a blank control.

Cell migration. A Transwell assay was performed to quantify *in vitro* migration of HUVECs. Cells pretreated with conditional medium or normal medium for 48 h were digested with trypsin containing 0.25% ethylenediaminetetraacetic acid (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Following centrifugation (1,200 x g, 6 min, 4˚C), cells were diluted to $5x10^5$/ml, and then seeded in the upper chamber in serum-free medium. Medium containing 20% FBS was added to the lower chamber. Following a 24-h incubation at 37˚C, in a 5% CO_2 incubator for 24 h. Then, cells were fixed with 4% paraformaldehyde for 20 min at 4˚C, and the cells that had not migrated were removed using a cotton swab. The migrated cells were stained with 0.05% crystal violet for 20 min at room temperature. Ffollowing two washes for 3 min, the number of migrated cells were observed under an inverted microscope, photographed and the migration rate was calculated.

In vitro angiogenesis assay. Matrigel (cat. no. 356237; Corning Incorporated, Corning, NY, USA) was added to a pre-cooled 96-well plate and placed in the incubator for 30 min to allow Matrigel solidification. Then, 100 μl cells ($2x10^5$ cell/ml) were added to each well. To confirm the role of AKT and β-catenin in angiogenesis, LY294002 (1 μM; cat. no. S1105; Selleck Chemicals, Houston, TX, USA) and KYA1797K (0.75 μM; cat. no. S8327; Selleck Chemicals) were added to the medium. Cells were imaged after 6 h and the total length, number of branches, and mean mesh size was calculated to examine the angiogenesis ability of HUVECs using imageJ software (version 1.4; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data are presented as the mean ± standard deviation of at least three independent experiments. The data were analyzed using the GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical analyses were performed with Student's t-test or one-way analysis of variance with the least significant difference post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression level of miR-24 in glioma cells. Following transfection with miR-24 mimics, inhibitors or NC for 48 h, the miR-24 expression levels in U251 cells were detected by RT-qPCR (Fig. 1). After 48 h, miR-24 was significantly upregulated by miR-24 mimics (Fig. 1A) and significantly downregulated by miR-24 inhibitors (Fig. 1B). miR-24 was also detected in the culture medium (Fig. 1C). miR-24 in the conditional medium was not significantly changed. Thus, the direct effect of miR-14 on ECs may be negligible. Subsequently, the culture medium was collected and prepared for HUVEC culture.

Expression level of VEGFA in culture medium from miR-24 mimic- or inhibitor-transfected U251 cells. VEGFA was detected in the culture medium (Fig. 1D). Results demonstrated that VEGFA was significantly increased in the culture medium from miR-24 mimic-transfected U251 cells, whereas it was significantly decreased in the culture medium from miR-24 inhibitor-transfected U251 cells. As miR-24 in the culture medium was not significantly changed, miR-24 may not influence the response to VEGFA in ECs. The VEGFA expressed by U251 may promote EC viability and angiogenesis.

Effect of conditional medium from miR-24 mimic- or inhibitor-transfected U251 cells on cell viability of HUVECs. To detected whether the overexpression of miR-24 in U251 cells affects the cell viability of HUVECs, the conditional medium samples collected from miR-24 mimic- or inhibitor-transfected U251 cells were used to culture the HUVECs for 24 h and cell viability was examined by MTT (Fig. 2). The conditional medium from miR-24 mimic-transfected U251 cells demonstrated significantly increased cell viability of HUVECs (Fig. 2A). By contrast, the conditional medium from miR-24 inhibitor-transfected U251 cells exhibited significantly decreased cell viability of HUVECs (Fig. 2B).

Effect of conditional medium from miR-24 mimic- or inhibitor-transfected U251 cells on cell migration of HUVECs. To detected whether the overexpression of miR-24 in U251 cells affects the cell migration of HUVECs, the conditional medium samples collected from miR-24 mimic- or inhibitor-transfected U251 cells were used to culture the HUVECs for 24 h, and cell migration was examined by Transwell assay (Fig. 3). The conditional medium from miR-24 mimic-transfected U251 cells exhibited significantly increased cell migration of HUVECs (Fig. 3A and B). By contrast, the conditional medium from miR-24 inhibitor-transfected U251 cells demonstrated significantly decreased cell migration of HUVECs (Fig. 3A and C).

Effect of conditional medium from miR-24 mimic- or inhibitor-transfected U251 cells on angiogenesis of HUVECs. To further detect whether the overexpression of miR-24 in U251 cells affects the angiogenesis of HUVECs, the conditional medium collected from miR-24 mimic- or inhibitor-transfected U251 cells were used to culture the HUVECs for 24 h, and the tube formation ability was examined (Fig. 4). The conditional medium from miR-24 mimic-transfected U251 cells demonstrated significantly increased tube formation of HUVECs (Fig. 4A-D). By contrast, the conditional medium from miR-24 inhibitor-transfected U251 cells exhibited significantly decreased tube formation of HUVECs (Fig. 4A and E-G).

Regulatory mechanism of angiogenesis in conditional medium from miR-24 mimic- or inhibitor-transfected U251 cells. The regulatory mechanism of angiogenesis in HUVECs from conditional medium from miR-24 mimic- or inhibitor-transfected U251 cells was investigated (Figs. 5-7). The mRNA expression levels of VEGF, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), TGF-β, matrix

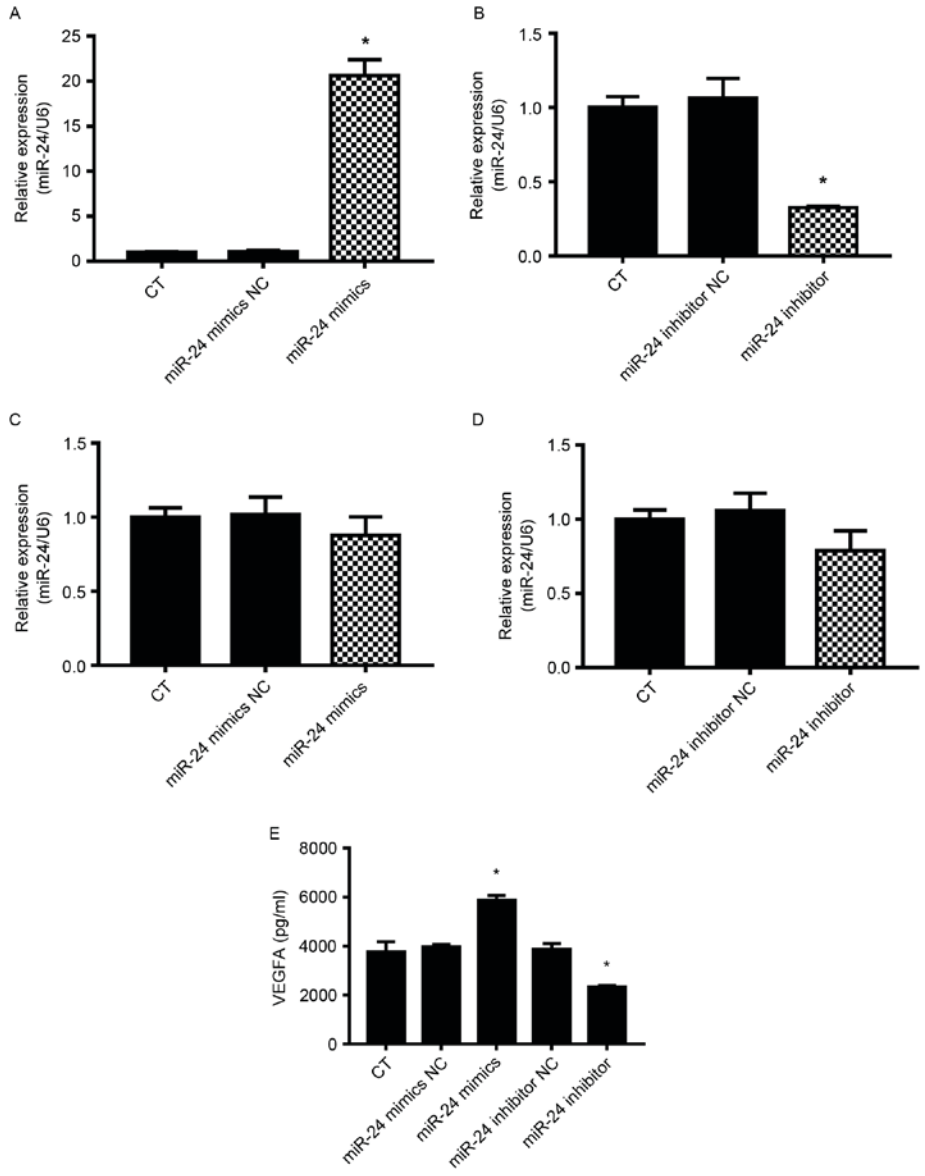

Figure 1. Expression levels of miR-24 in U251 glioma cells. Following transfection with miR-24 (A) mimics, (B) inhibitors or NC for 48 h, miR-24 levels in the cell lysate were detected by reverse transcription-quantitative polymerase chain reaction. miR-24 expression levels were also detected in the culture medium following transfection with (C) mimics, (D) inhibitors or NC. (E) VEGFA was detected in the culture medium by ELISA. *P<0.05, **P<0.01 vs. NC. miR, microRNA; NC, negative control; CT, untransfected cells; VEGFA, vascular endothelial growth factor A.

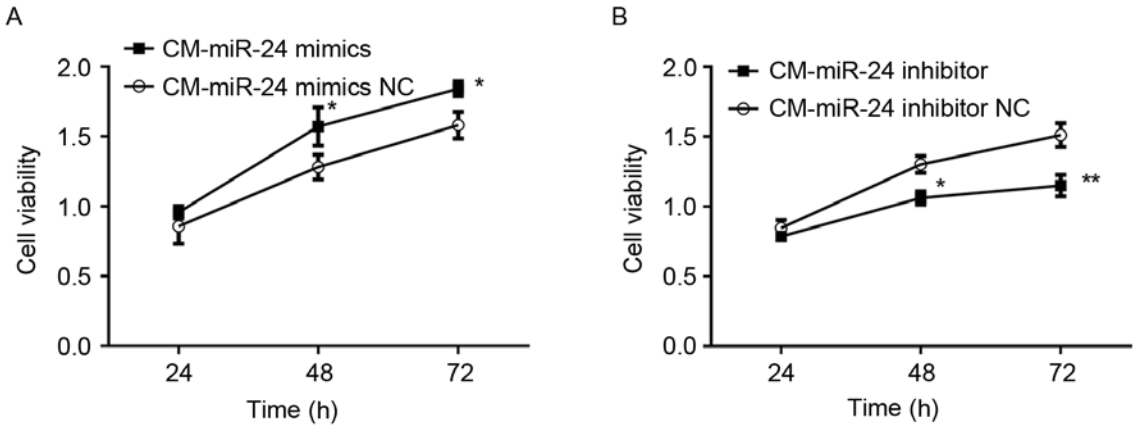

Figure 2. Effect of CM from miR-24 mimic- or inhibitor-transfected U251 cells on cell viability of HUVECs. The CM collected from miR-24 (A) mimic- or (B) inhibitor-transfected U251 were used to culture the HUVECs for 24, 48, and 72 h, and the cell viability was examined by MTT. The cell viability axes indicate the optical density. *P<0.05, **P<0.01 vs. NC. CM, conditional medium; miR, microRNA; HUVEC, human umbilical vein endothelial cell; NC, negative control.

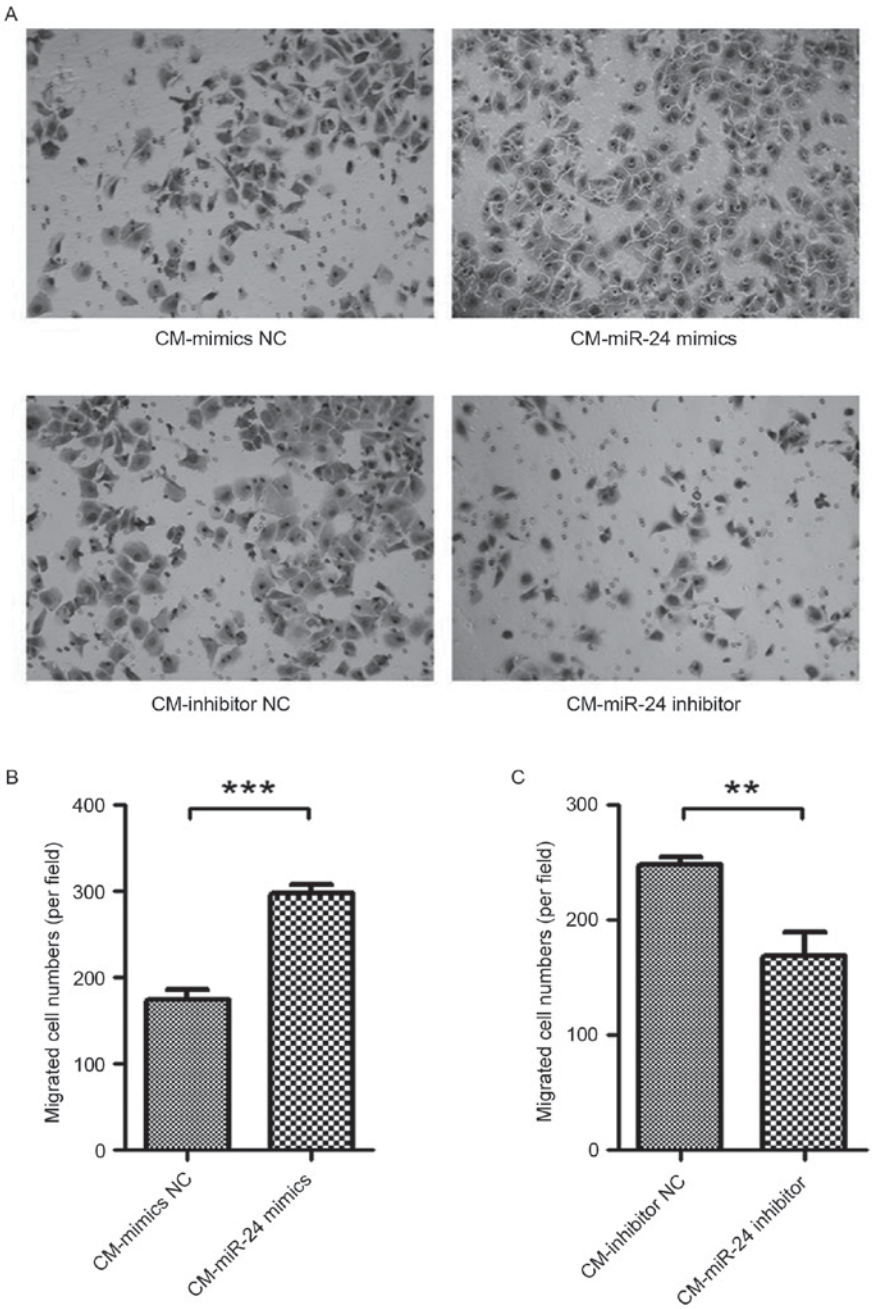

Figure 3. Effect of CM from miR-24 mimic- or inhibitor-transfected U251 on cell migration of HUVECs. The CM collected from miR-24 mimic- or inhibitor-transfected U251 were used to culture the human umbilical vein endothelial cells for 24 h, and the cell migration was examined by Transwell assay. (A) Migrated cells (magnification, x200). (B) Cell numbers in CM-mimics NC and CM-miR-24 mimics groups. (C) Cell numbers in CM-inhibitor NC and CM-miR-24 inhibitor groups. **P<0.01, ***P<0.001 vs. NC. CM, conditional medium; miR, microRNA; NC, negative control.

metalloproteinase (MMP)-2 and MMP-9 in U251 cells were significantly increased by miR-24 mimics (Fig. 5A-F), and were significantly decreased by miR-24 inhibitors (Fig. 5G-L). Western blot detection confirmed the increase of VEGF and TGF-β protein expression levels in U251 by miR-24 mimics (Fig. 5M), and the decrease of VEGF and TGF-β protein expression levels in U251 by miR-24 inhibitors (Fig. 5N). It was proposed that the change of VEGF and TGF-β expression levels in U251 by miR-24 may associated with the angiogenesis of HUVECs.

Subsequently, the expression levels of angiogenesis-associated genes in HUVECs were further detected (Fig. 6). The mRNA expression levels of VEGF, TGF-β, MMP-2 and MMP-9 in HUVECs were significantly increased by treatment of conditional medium from miR-24 mimic-transfected U251 cells (Fig. 6A-E), but were significantly decreased by treatment of conditional medium from miR-24 inhibitor-transfected U251 cells (Fig. 6F-J). However, the expression level of bFGF mRNA was not significantly changed by the conditional mediums from miR-24 mimic- and inhibitor-transfected U251 cells.

Western blot detection confirmed the increase of VEGF and TGF-β protein expression levels in U251 in the conditional medium from miR-24 mimic-transfected U251 cells (Fig. 6K), and the decreased protein expression level of VEGF and TGF-β in U251 cells by conditional medium from miR-24

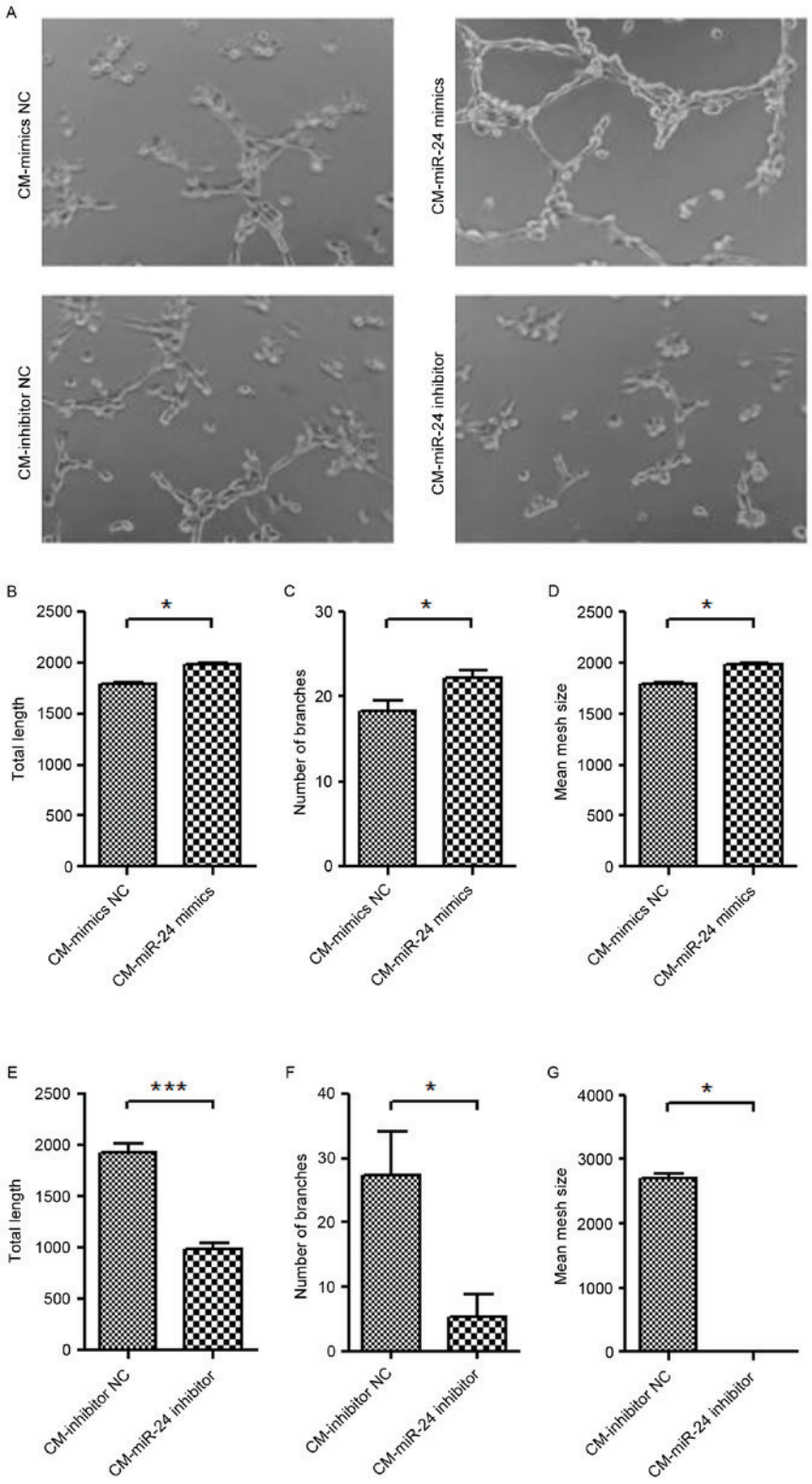

Figure 4. Effect of conditional medium from miR-24 mimic- or inhibitor-transfected U251 cells on angiogenesis of HUVECs. The CM collected from miR-24 mimic- or inhibitor-transfected U251 cells was used to culture the HUVECs for 24 h, and the tube formation was examined. (A) Tube formation (magnification, x100). Quantification data of the mimics groups, including (B) total length, (C) number of branches and (D) mean mesh size. Quantification data of inhibitor groups, including (E) total length, (F) number of branches and (G) mean mesh size. *P<0.05, ***P<0.001 vs. NC. miR, microRNA; HUVEC, human umbilical vein endothelial cell; CM, conditional medium; NC, negative control.

inhibitor-transfected U251 cells (Fig. 6L). It was further indicated that the changed expression levels of VEGF and TGF-β in U251 cells by miR-24 may be associated with the angiogenesis of HUVECs.

To investigate intracellular signaling, the expression of p-AKT, t-AKT and β-catenin were detected in HUVECs by treatment of conditional medium from miR-24 inhibitor-transfected U251 cells (Fig. 7A-H). p-AKT, t-AKT and β-catenin

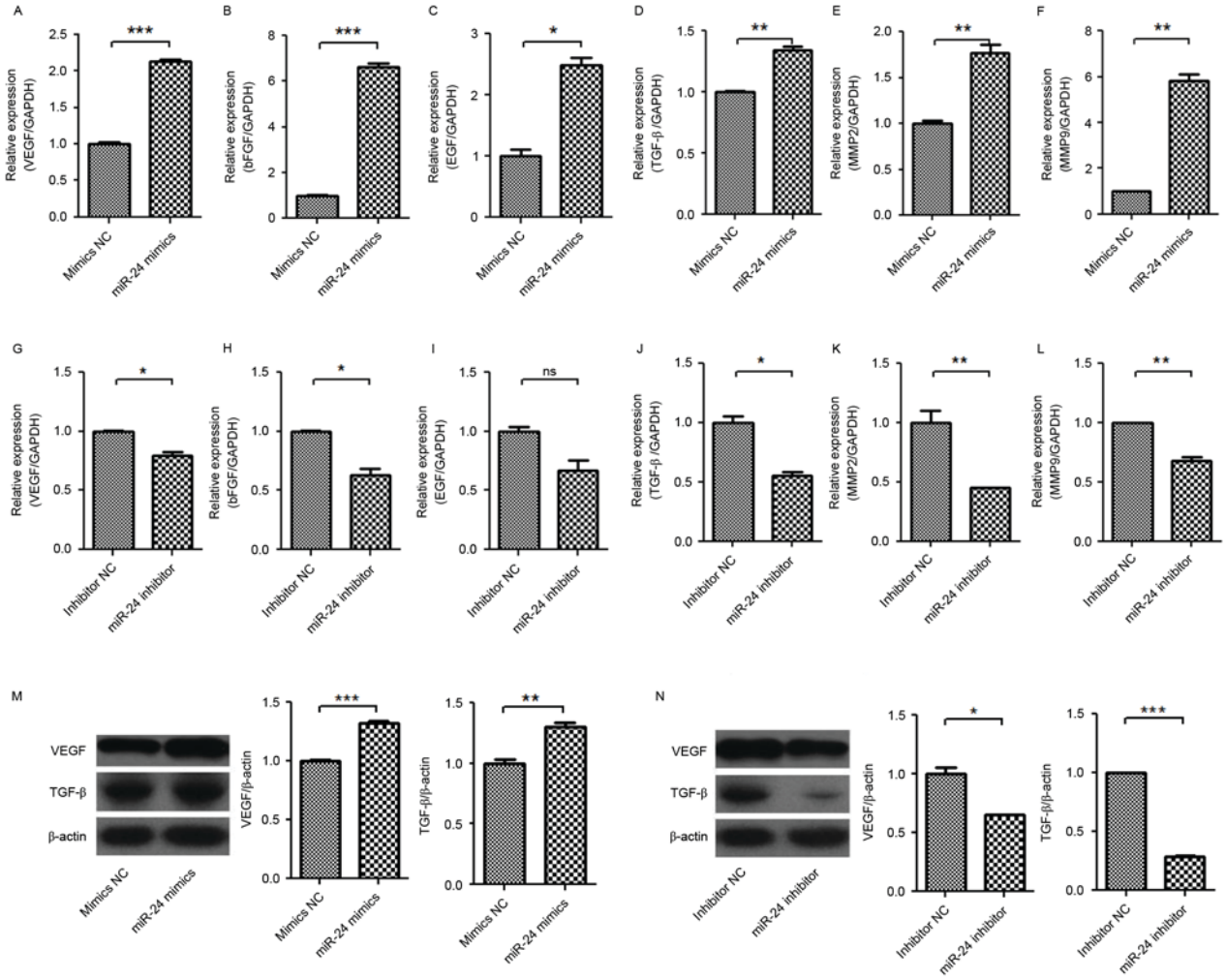

Figure 5. Effect of miR-24 on mRNA expression levels of VEGF, bFGF, EGF, TGF-β, MMP-2 and MMP-9, and protein expression levels of VEGF and TGF-β in U251 cells. The U251 cells were transfected with miR-24 mimics or inhibitors. Following 48 h, the mRNAs and protein was detected by reverse transcription-quantitative polymerase chain reaction and western blot analysis. Expression levels of (A) VEGF mRNA, (B) bFGF mRNA, (C) EGF mRNA, (D) TGF-β mRNA, (E) MMP-2 mRNA and (F) MMP-9 mRNA induced by miR-24 mimics. Expression levels of (G) VEGF mRNA, (H) bFGF mRNA, (I) EGF mRNA, (J) TGF-β mRNA, (K) MMP-2 mRNA, (L) MMP-9 mRNA inhibited by miR-24 inhibitors. Expression levels of VEGF and TGF-β (M) induced by mir-24 mimics and (N) inhibited by miR-24 inhibitors. *P<0.05, **P<0.01; ***P<0.001 vs. NC. ns, not significant; miR, microRNA; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; TGF-β, transforming growth factor β; MMP, matrix metalloproteinase; NC, negative control.

expression levels in HUVECs were upregulated by conditional medium from miR-24 mimic-transfected U251 cells, and were decreased by conditional medium from miR-24 inhibitor-transfected U251 cells. The roles of AKT and β-catenin in angiogenesis were confirmed by using LY294002 and KYA1797K in HUVECs, respectively (Fig. 7I). Results indicated that the miR-24 mimic-induced angiogenesis was inhibited by LY294002 and KYA1797K. Thus, AKT and β-catenin are involved in angiogenesis in gliomas.

Discussion

The current study demonstrated that enforced expression of miR-24 in U251 cells promotes cell viability and angiogenesis of HUVECs, and increased the expression levels of angiogenesis-associated factors, including VEGF, TGF-β, MMP-2 and MMP-9. By contrast, reduced expression of miR-24 in U251 cells may inhibit cell viability and angiogenesis of HUVECs, and downregulate expression levels of angiogenesis-associated factors, including VEGF, TGF-β, MMP-2 and MMP-9. The miR-24 in U251 cells may be important in the angiogenesis of HUVECs via VEGF and TGF-β, and the intracellular signaling AKT and β-catenin involved in this process.

It was demonstrated that the expression level of VEGF mRNA in patients with grade III and IV gliomas was significantly higher than that in patients with grade I and II gliomas, and the expression level of VEGF mRNA in patients with grade I and II gliomas was significantly higher than that in normal brain tissues (25-27). In addition, the cell viability, migration and invasion, and angiogenesis of gliomas may be inhibited by inhibition of the pituitary tumor transforming gene that was positively associated with the glioma grade and tumor microvessel density (28), and was considered as an important target of glioma antiangiogenic therapy. It was also demonstrated that certain angiogenic chemokines secreted by tumor cells may directly effect vascular ECs, which induces angiogenesis (29). Furthermore, angiogenesis is regulated by the receptor of those chemokines, including vascular endothelial

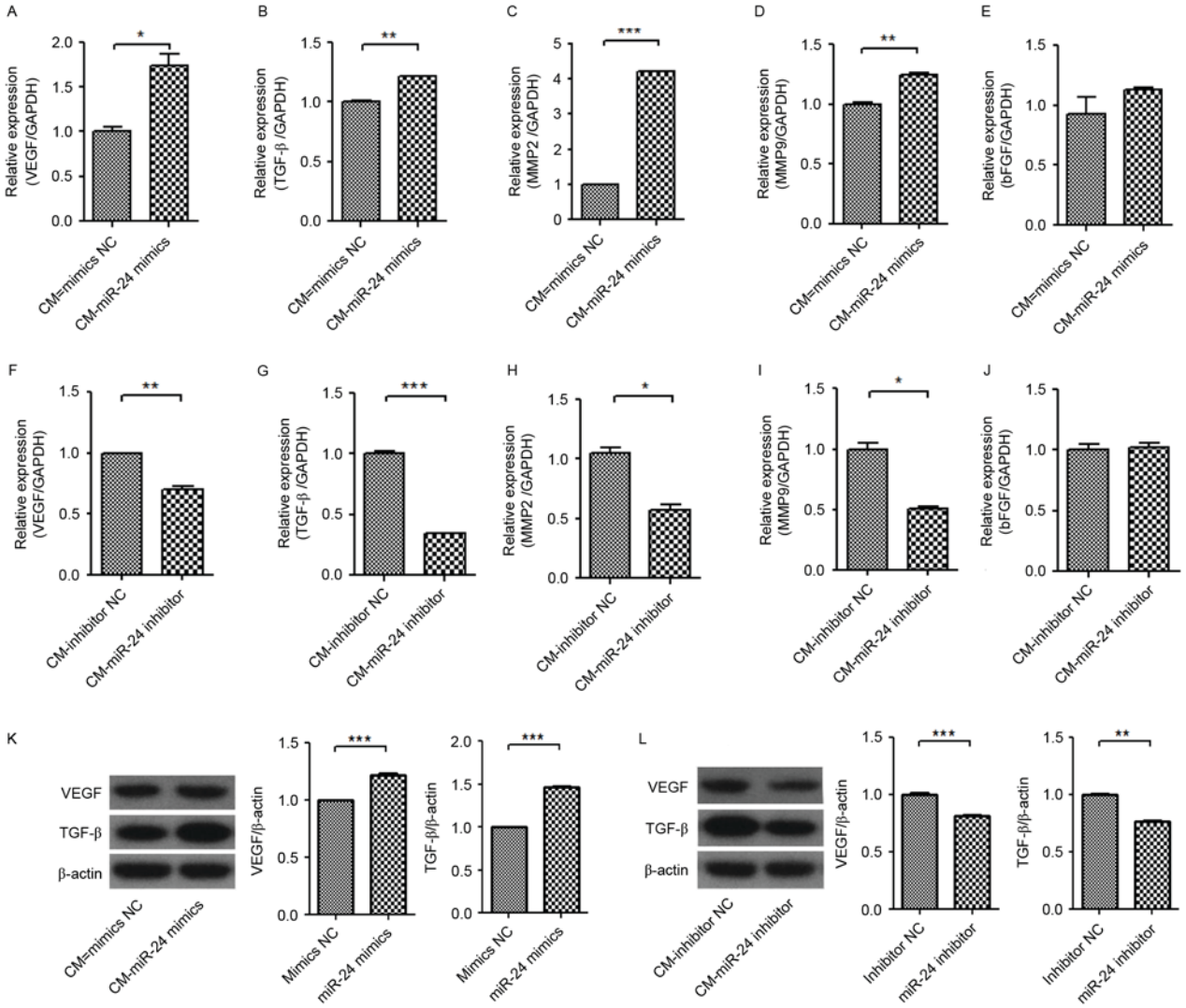

Figure 6. Effect of CM from miR-24 mimic- or inhibitor-transfected U251 cells on mRNA expression levels of VEGF, TGF-β, MMP-2, MMP-9 and bFGF and protein expression levels of VEGF and TGF-β in HUVECs. The U251 cells were transfected with miR-24 mimics or inhibitors. Following treatment with the CMs for 24 h, the mRNAs and proteins were detected by reverse transcription-quantitative polymerase chain reaction and western blot analysis. mRNA expression levels of (A) VEGF, (B) TGF-β, (C) MMP-2, (D) MMP-9 and (E) bFGF induced by miR-24 mimics. mRNA expression levels of (F) VEGF, (G) TGF-β, (H) MMP-2, (I) MMP-9 and (J) bFGF inhibited by miR-24 inhibitors. Protein expression levels of VEGF and TGF-β (K) induced by mir-24 mimics and (L) inhibited by miR-24 inhibitors. *P<0.05, **P<0.01; ***P<0.001 vs. NC. CM, conditional medium; miR, microRNA; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; TGF-β, transforming growth factor β; MMP, matrix metalloproteinase; NC, negative control.

growth factor receptor, epidermal growth factor receptor and platelet-derived growth factor receptor. Currently, studies have demonstrated that in the newly diagnosed grade III malignant glioma patients, ~61% of patients achieved a good outcome following treatment with Avastin (VEGF mAb) combined with irinotecan in the second phase of clinical trials, and adverse reactions were decreased compared with the use of irinotecan alone (30). shRNA plasmids encapsulated with urokinase-type plasminogen activator (uPA) and the corresponding receptor (uPAR), and metalloproteinases, such as MMP-9, significantly inhibit angiogenesis and tumor growth in mice (31). Similarly, in animal models of transplanted tumors, injection of a VEGF siRNA plasmid significantly inhibits tumor angiogenesis (32).

miR-24 promotes tumors and angiogenesis in various types of cancer, such as pancreatic carcinoma (33). miR-24 expression was downregulated in glioma samples and glioma cells (19,20,34). Overexpression of miR-24-3p may promote cell proliferation, as observed by MTT assay (19). The suppression of miR-24 expression inhibits cell proliferation and invasion, indicating that that miR-24 acts as an oncogene in gliomas (20). Certain genes closely associated with angiogenesis (such as VEGF, bFGF, EGF, TGF-β, MMP-2 and MMP-9) were detected. It was found that enforced expression of miR-24 in U251 cells significantly increased the mRNA expression levels of VEGF, bFGF, EGF, TGF-β, MMP-2 and MMP-9 in U251 cells, as well as the VEGF and TGF-β protein expression levels in U251 cells. By contrast, reduced expression of miR-24 in U251 cells significantly decreased the mRNA expression levels of VEGF, bFGF, EGF, TGF-β, MMP-2 and MMP-9 in U251 cells, as well as the VEGF and TGF-β protein expression levels in U251 cells. To detected whether the dysregulation of miR-24 in glioma cells promotes microvascular proliferation of ECs and investigate its potential underlying mechanism, conditioned media was used to investigate the secretion of

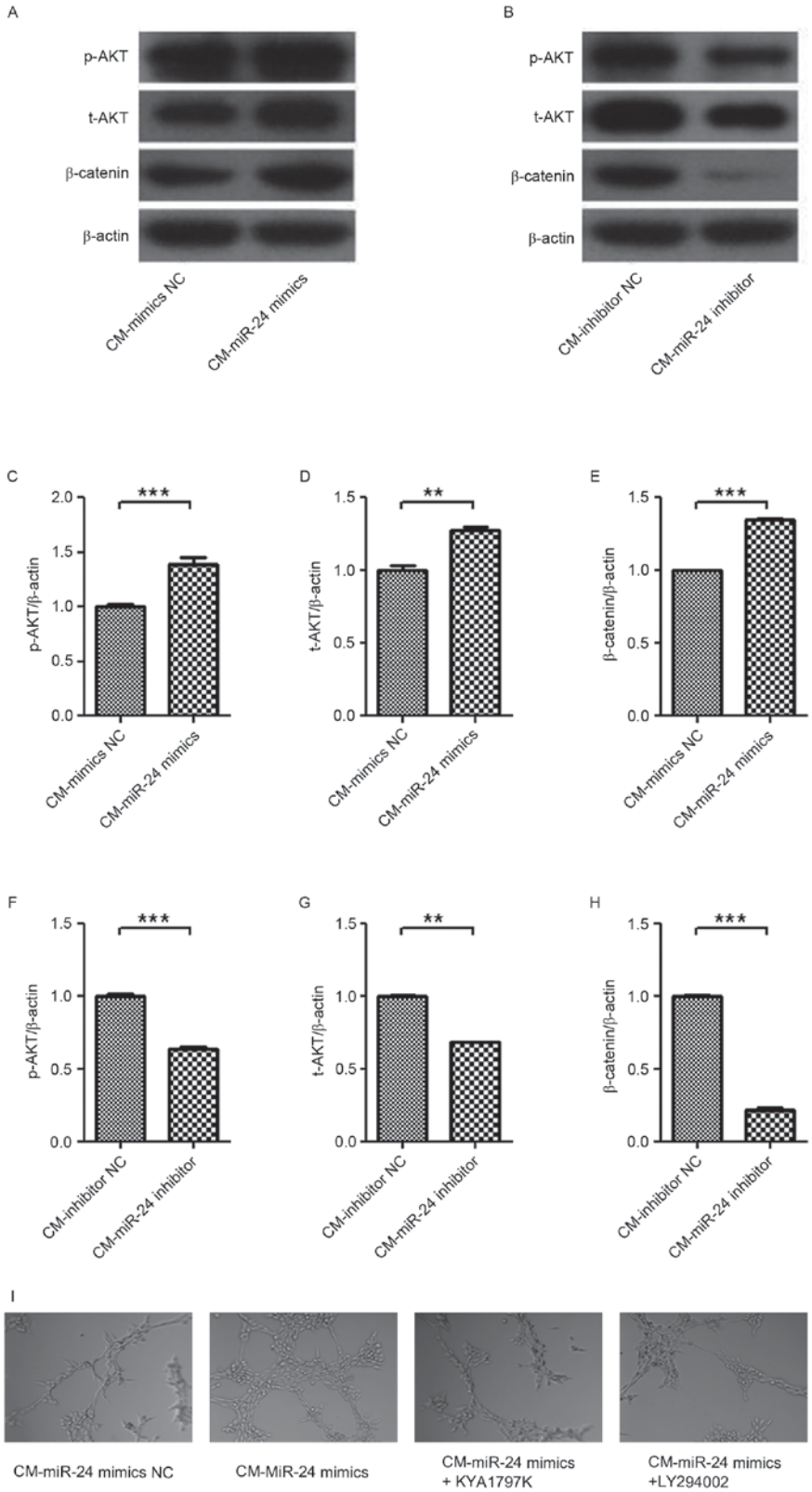

Figure 7. Effect of CM from miR-24 mimic- or inhibitor-transfected U251 cells on the expression levels of p-AKT, t-AKT and β-catenin signaling in HUVECs. The U251 cells were transfected with miR-24 mimics or inhibitors, and then the CM was collected. Following treatment with the CM for 24 h, the expression levels of p-AKT, t-AKT and β-catenin in HUVECs were detected by western blotting: (A) CM-mimics and (B) CM-inhibitors, and quantification data for (C-E) CM-mimics and (F-H) CM-inhibitors. The roles of AKT and β-catenin in angiogenesis were confirmed using LY294002 and KYA1797K, respectively, in HUVECs. (I) Tube formation (magnification, x100). **P<0.01; ***P<0.001 vs. NC. CM, conditional medium; HUVEC, human umbilical vein endothelial cell; NC, negative control.

pro-angiogenic molecules (such as VEGF, bFGF, EGF, TGF-β, MMP-2, MMP-9) following miR-24 transfection. All these pro-angiogenic molecules have demonstrated critical roles in tube formation of HUVECs. VEGFA was significantly

increased in the culture medium from miR-24 mimic-transfected U251 cells, whereas it was significantly decreased in the culture medium from miR-24 inhibitor-transfected U251 cells. As the miR-24 in the culture medium was not significantly changed, miR-24 may not influence the response to VEGFA in ECs. Therefore, miR-24 may contribute to angiogenesis in glioma via upregulation of VEGF and TGF-β expression levels in U251 cells. This was further confirmed using the HUVECs by treatment of the of conditional medium from miR-24 mimic- or inhibitor-transfected U251 cells. Although the bFGF mRNA was not significantly changed by the conditional mediums from miR-24 mimic- and inhibitor-transfected U251 cells, conditional medium from miR-24 mimic-transfected U251 cells significantly increased the mRNA expression levels of VEGF, TGF-β, MMP-2 and MMP-9, while the conditional medium from miR-24 inhibitor-transfected U251 cells significantly decreased. Thus, miR-24 contributes to angiogenesis in gliomas via upregulation of VEGF and TGF-β expression in U251 cells.

AKT and β-catenin signaling are two important signaling pathways involved in the angiogenesis of HUVECs. p-AKT, t-AKT and β-catenin expression levels were upregulated in conditional medium from miR-24 mimic-transfected U251 cells, and were decreased in the conditional medium from miR-24 inhibitor-transfected U251 cells. The miR-24 mimic-induced angiogenesis was inhibited by LY294002 and KYA1797K, indicating that AKT and β-catenin are involved in angiogenesis in gliomas.

In conclusion, miR-24 may contribute to the angiogenesis in gliomas via upregulation of VEGF and TGF-β expression levels in U251 cells and the intracellular AKT and β-catenin signaling pathway. However, the present study was only performed *in vitro* using one cell line, the human U251 glioma cell line. A further study with more cell lines and an *in vivo* investigation is required in future. However, the current findings may contribute to the therapy of gliomas.

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