Int J Clin Exp Pathol 2014;7(3):1077-1084
www.ijcep.com /ISSN:1936-2625/IJCEP1312082

Original Article

VE-statin/Egfl7 siRNA inhibits angiogenesis in malignant glioma *in vitro*

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Received December 25, 2013; Accepted January 27, 2014; Epub February 15, 2014; Published March 1, 2014

Abstract: This study investigated the role of VE-statin/Egfl7 and its mechanism in angiogenesis in malignant glioma. Transwell culture plates were used to establish an U251-HUVEC co-culture system, which was used to mimic the interaction between malignant glioma and endothelial cells. Lentiviral vectors expressing VE-statin/Egfl7 siRNA were constructed, and U251 cells and HUVECs were transfected to inhibit VE-statin/Egfl7 expression. The proliferation, adherence, migration, and lumen formation of endothelial cells were assayed to investigate the influence of VE-statin/Egfl7 on angiogenesis in malignant glioma *in vitro*. Data showed that HUVEC growth was temporarily slowed after silencing the VE-statin/Egfl7 gene but rapidly returned to normal. Although endothelial cell migration was not influenced, cell adherence was markedly inhibited. Furthermore, the endothelial cells failed to generate a capillary-like lumen after VE-statin/Egfl7 gene silencing. Therefore, it can be concluded that VE-statin/Egfl7 may regulate the adherence of endothelial cells, thus playing an important role in endothelium-induced lumen formation during angiogenesis in malignant glioma.

Keywords: Epidermal growth factor-like domain 7, malignant glioma, angiogenesis, RNA interference

Introduction

Glioma, a malignancy of glial cells derived from neuroectoderm, is the most common type of intracranial tumor. Although great progress has been achieved in past years, the prognosis of malignant glioma patients remains poor, with a mean survival time of only 12-15 months. The growth and invasion of glioma cells are dependent on angiogenesis, and a continuous increase in blood vessels has been regarded as an important malignant characteristic of glioma [1]. Although the mechanisms underlying angiogenesis in cancers are complex, identifying targets to control angiogenesis is clinically important. Epidermal growth factor-like domain 7 (also known as VE-statin/Egfl7) is an angiogenesis-related factor derived from endothelial cells that is highly expressed during embryonic development. In particular, VE-statin/Egfl7 plays a crucial regulatory role in the angiogenesis that occurs during this stage. However, VE-statin/Egfl7 is not or is only weakly expressed in most human tissues, with the exception of tissues that are rich in blood vessels, such as the lung, ovary, and uterus [2]. VE-statin/Egfl7 is also highly expressed in both liver cancer and malignant glioma, and increasing evidence shows that VE-statin/Egfl7 expression is reactivated in multiple malignancies [3, 4]. The present study aimed to investigate the role of VE-statin/Egfl7 in the angiogenesis of malignant glioma using a small interfering RNA (siRNA) technique to elucidate the role of VE-statin/Egfl7 and its mechanism in glioma.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) and a human astrocytoma cell line (U251; WHO grade IV pleomorphic glioblastoma) were maintained in the Xiangya Central Laboratory of Central South University. These cells were cultured in RPMI 1640 (CELLWAY-LAB, Luoyang, China) containing 12% fetal bovine serum (FBS) at 37°C in an environment with 5% CO_2.

Reagents and instrumentation

TRIzol reagent (Invitrogen, USA); reagents for RT-PCR (TAKARA); FBS, RPMI 1640, and Opti-MEM (Gibco); MTT and crystal violet (Sigma); a plasmid extraction kit (Qiagen); primers (Shanghai Genechem Biotech Co., Ltd.); a fluorescence microscope (Olympus); protein electrophoresis equipment and protein transfer equipment (Shanghai Tanon Biotech Co., Ltd.); an inverted phase-contrast microscope (Nikon TMS-F); Transwell culture plates (Costar, USA); and multiwell plates (Corning, USA) were used in the present study.

Establishment of an U251-HUVEC co-culture system using Transwell culture plates

In brief, Transwell chambers were added to a 24-well plate, and a semipermeable polycarbonate membrane was added to each well. The lower-chamber and upper-chamber solutions were added to the respective chambers, which were then separated by a polycarbonate membrane. Next, HUVECs and U251 cells were seeded into the upper and lower chambers, respectively. The semipermeable membrane allows the interchange of components between the upper and lower media, which is helpful for investigating the interaction between cells in the two chambers. When the pore size is <3.0 μm (generally, the pore size is 0.4 μm), cells cannot migrate across the membrane, but secreted substances can. When the pore size is >3.0 μm, cells in the upper chamber can migrate across the membrane in the presence of chemokine secreted by cells in the lower chamber.

Treatments

The cells were divided into three groups: (1) a normal negative-control group (CC), in which no treatment was performed; (2) U251 cells transfected with a scrambled siRNA sequence (NC) and co-cultured with HUVECs; and (3) U251 cells transfected with VE-statin/Egfl7 siRNA (KD) and co-cultured with HUVECs.

Construction of VE-statin/Egfl7-siRNA lentiviral vectors and cell infection

In our previous study, a target sequence in the VE-statin/Egfl7 gene was identified for RNA interference, and siRNA lentiviral vectors expressing a green fluorescent protein (GFP)-conjugated reporter gene were successfully constructed. These vectors could effectively inhibit VE-statin/Egfl7 expression in both U251 cells and HUVECs. The construction of the VE-statin/Egfl7-siRNA lentiviral vectors and cell infection were performed as previously reported [5, 6].

Detection of HUVEC proliferation (MTT assay)

Cells in the logarithmic growth phase were harvested, and the cell density was adjusted. The cells were then seeded into 24-well plates. HUVECs were added to the lower chamber of 24-well plates (6×10^3 cells/well), and U251 cells were added to the upper chamber of 24-well plates (1×10^5 cells/well). The two types of cells were maintained independently at 37°C in an environment with 5% CO_2. When the cell confluence reached 30%, co-culture was performed using U251 cells that had previously been infected with lentivirus. MTT assays were performed after culturing for 24 h, 48 h, 72 h, 96 h, and 5 d. Briefly, the supernatant was removed, and fresh RPMI 1640 (480 μl) and 0.5% MTT solution were added sequentially, followed by incubation for 4 h. The supernatant was removed, and the polycarbonate membrane was collected and placed in a 96-well plate. DMSO (150 μl/well) was added, followed by incubation for 10 min with continuous shaking to dissolve any crystals, after which the polycarbonate membrane was removed. Blank controls were also included (DMSO alone). Five wells were used for each group, and the optical density (OD) was measured at 492 nm using a microplate reader.

Detection of HUVEC adherence and migration (crystal violet method)

After transfection for 72 h, HUVECs were harvested and added to the upper chamber (200 μl/well; 2×10^4 cells/well), to which a collagen-coated semipermeable membrane (pore size: 0.4 μm) was also added. The cells were incubated for 30 min, and the supernatant and suspended cells were then removed. After washing twice in 0.01 M PBS, the cells were fixed in 4% paraformaldehyde for 10 min. Next, the supernatant was removed, and the membrane was dried. Crystal violet (0.1%, 0.6 ml/well) was added and incubated at room temperature for 20 min. After removal of the staining solution, the upper chamber was washed with distilled water and collected. The outside of the polycar-

bonate membrane was air-dried. Prior to measurement, the upper chambers were placed in 24-well plates, and 33% acetic acid was added (0.6 ml) for decolorization, followed by vortexing. Five wells were used for each group, and a blank control group was also included (33% acetic acid alone). Then, 150 μl of decolorization solution was added to each well of the 96-well plates, and the OD was measured at 570 nm.

A polycarbonate membrane (pore size: 8.0 μm) was again used in the detection of cell migration. HUVECs were added to the upper chambers (200 μl; 1×10^5 cells/chamber), and the cells that had migrated were counted after 24 h. In particular, the medium was removed, and the cells on the inside of the polycarbonate membrane were collected using a swab. After washing in 0.01 M PBS, the membrane was fixed in 4% paraformaldehyde for 10 min. Crystal violet staining was performed as described above for the detection of adherence.

Detection of HUVEC lumen formation capability

Rat tail collagen was prepared and mixed with 10× RPMI 1640, 0.1 N sodium hydroxide, and FBS at a volume ratio of 7:1:1:1 in a sterilized flask, and the pH was adjusted to 7.4. Cells in the logarithmic growth phase were harvested and maintained in 24-well plates (pore size: 0.4 μm), and the cell density was adjusted. HUVECs were then added to the lower chamber of 24-well plates (4×10^4 cells/well), and U251 cells were added to the upper chamber of 24-well plates (4×10^4 cells/well) for co-culture. After lentivirus infection as described above, the cells were incubated at 37°C in an environment with 5% CO_2. When the HUVEC confluence reached nearly 100%, and single-layer cells were observed, collagen was added to the lower chamber containing HUVECs (300 μl/well), followed by incubation at 37°C in an environment with 5% CO_2. The lumen formation of HUVECs was then observed under a microscope.

Statistical analysis

A statistical analysis was performed with SPSS version 13.0, and the data were expressed as the mean ± standard deviation ($\bar{x}\pm s$). A value of $P<0.05$ was considered statistically significant. Comparisons of the means among multiple groups were performed with one-way analysis of variance (ANOVA) and a subsequent SNK-*q* test. Moreover, images were analyzed using Image-Pro Plus 6.0 software.

Results

U251 cells transfected with VE-statin/Egfl7 siRNA and co-cultured with HUVECs

U251 cells were transfected with lentivirus expressing VE-statin/Egfl7 siRNA and then co-cultured with HUVECs for 48 h. When examined under an inverted phase-contrast fluorescence microscope, the infected cells showed a strong green fluorescence; the transfection efficiency was >80%. In contrast, no fluorescence was observed in the control group. Moreover, the fluorescence quantitative PCR results showed that VE-statin/Egfl7 expression was inhibited by >80% in comparison with the control group (**Figure 1**).

Detection of HUVEC proliferation

Royal blue formazan crystals were observed after the addition of MTT solution, and the OD was measured using a microplate reader after adding DMSO to dissolve the crystals. The proliferation of HUVECs in the KD group was slower than that in the other two groups and was observed after up to 4 d of culturing ($P<0.01$). After 5 d of culturing, HUVEC proliferation returned to the baseline level and was comparable with proliferation in the control group ($P>0.05$). Additionally, there was no significant difference in the proliferation of HUVECs between the CC and the NC groups ($P>0.05$) (**Figure 2A-C** and **Table 1**).

Changes in HUVEC adherence and migration

In the CC and NC groups, the OD was significantly higher than the OD of the cells transfected with VE-statin/Egfl7 siRNA after culturing for 30 min ($P<0.01$), suggesting that the VE-statin/Egfl7 siRNA significantly inhibited the adherence of HUVECs. In contrast, VE-statin/Egfl7 siRNA had no influence on the migration of HUVECs, and the number of migrated cells was comparable among the three groups after 24 h ($P>0.05$) (**Figure 3** and **Table 2**).

Lumen formation of HUVECs

After incubation with rat-tail collagen for 6 h, the morphology of HUVECs in the CC and NC

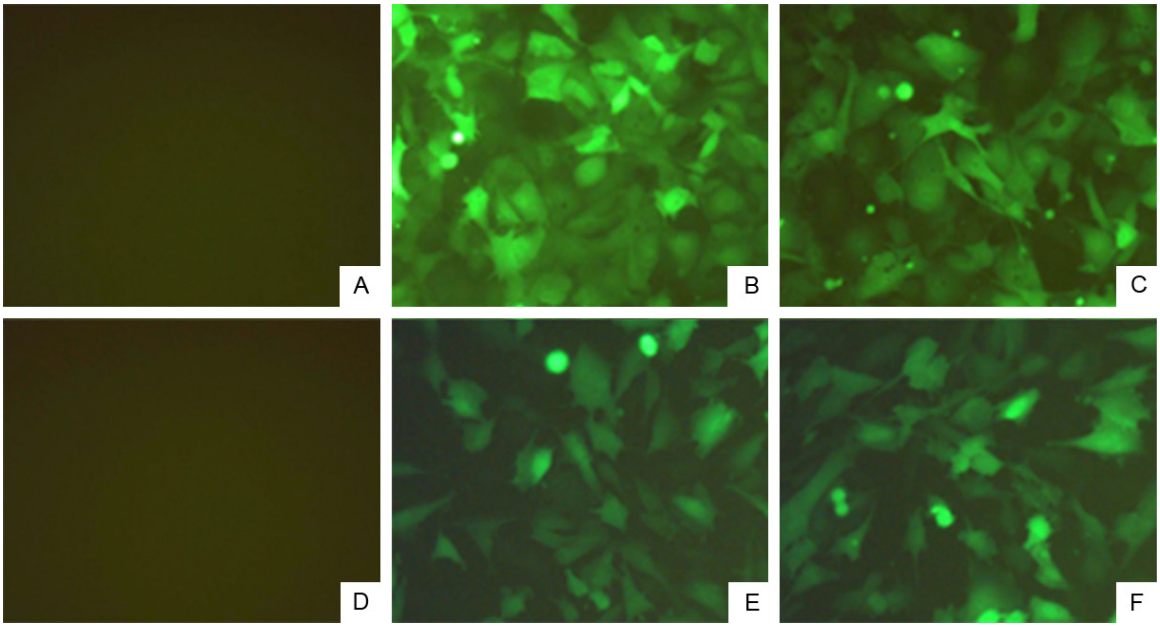

Figure 1. Fluorescence detection of HUVEC and U251 cells infected with the lentiviral expression vectors of siRNA targeting VE-statin/Egfl7. (A-C) Represented normal negative control HUVECs (A), the HUVECs infected with universal negative control lentiviral (B) or VE-statin/Egfl7-siRNA lentivirus (C) respectively; (D-F) Represented normal negative control U251 (D), the U251 infected with universal negative control lentiviral (E) or VE-statin/Egfl7-siRNA lentivirus (F) respectively.

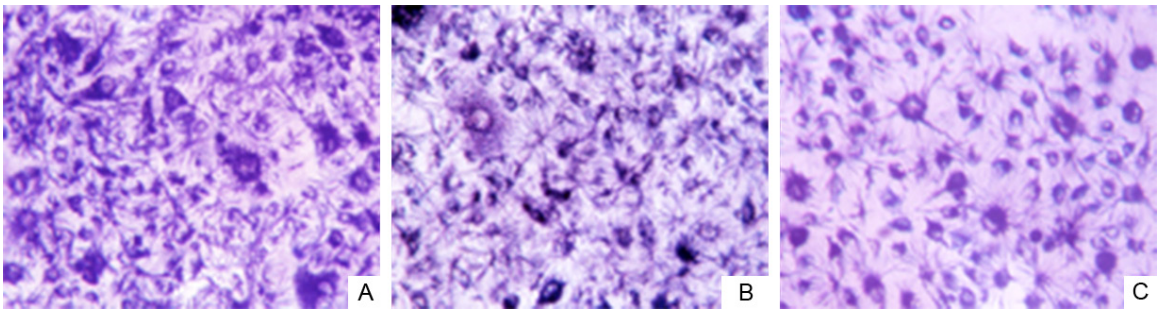

Figure 2. Effect of VE-statin/Egfl7 siRNA on the proliferation of HUVEC cells. (A, B) HUVECs proliferation at 48 h after incubation in group KD was significantly lower than that in the other two group, and this status continued to 4 d after incubation detected by cell proliferation assay (*, # vs. KD group, q=20.023, 16.686; 29.009, 26.034; 30.549, 28.672, $P<0.01$). Represented normal negative control HUVECs (A), the HUVECs infected with universal negative control lentiviral (B) or VE-statin/Egfl7-siRNA lentivirus (C) respectively.

groups was altered, with the cells becoming long and spindle shaped, and long pseudopodia were also observed. Moreover, the cells appeared extended, and the cell body became long and thin in comparison with the HUVECs without collagen. The transformed cells moved over time, and a clustering phenomenon was observed along the longitudinal axis. After 24 h of culture, capillary lumen-like structures formed by a single layer of HUVECs were observed and increased with time, becoming more evident after 3 d. In contrast to the control group, these lumen-like structures were not observed in the KD group. Images were captured at 24 h and quantified using Image-Pro Plus 6.0. The results showed that the area of the lumen-like structures was 0.599±0.052 and 0.587±0.036 in the CC and NC groups, respectively, and the number of lumens was 25.20±2.51 and 23.8±2.32 in the CC and NC groups, respectively, showing no significant differences ($P>0.05$).

Table 1. Effect of VE-statin/Egfl7 siRNA on the proliferation of HUVECs co-cultured with U251 cells (OD, $\bar{x}$±S)

Group	*n*	24 h	48 h	72 h	96 h	5 d
CC	5	0.093±0.004	0.244±0.008*	0.375±0.008*	0.534±0.011*	0.551±0.007
NC	5	0.090±0.007	0.230±0.014*	0.363±0.006*	0.523±0.013*	0.553±0.011
KD	5	0.082±0.008	0.160±0.002	0.258±0.012	0.355±0.015	0.513±0.012

Note: *vs KD group, *q*=20.023, 16.686; 29.009, 26.034; 30.549, 28.672, P<0.01.

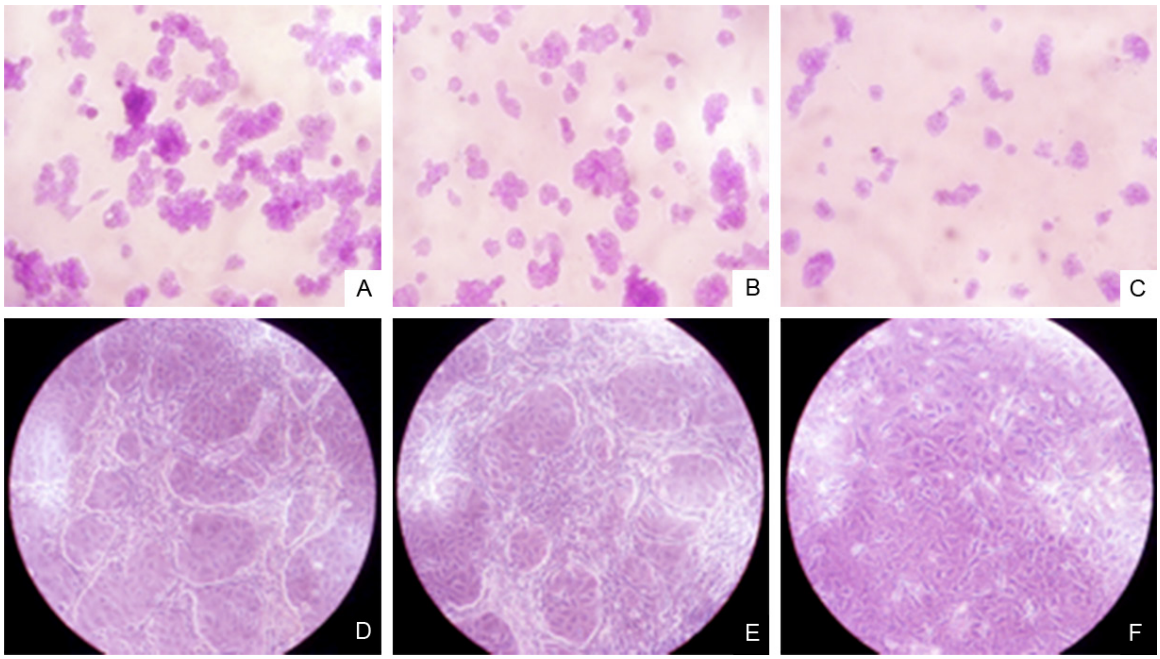

Figure 3. Effect of VE-statin/Egfl7 siRNA on the adhesion and tube formation of HUVEC cells. (A-C) Endothelial cell adhesion assay represented normal negative control HUVECs (A), the HUVECs infected with universal negative control lentiviral (B) or VE-statin/Egfl7-siRNA lentivirus (C) respectively; (D-F) Tube formation assay represented normal negative control HUVECs (D), the HUVECs infected with universal negative control lentiviral (E) or VE-statin/Egfl7-siRNA lentivirus (F) respectively.

Discussion

As with other solid tumors, the growth of glioma is highly dependent on angiogenesis. Our previous study showed that VE-statin/Egfl7 is highly expressed in vascular endothelial cells and malignant human glioma cells and is positively related to the degree of malignancy, cell proliferation, and angiogenesis [4]. Additionally, we found a high level of VE-statin/Egfl7 expression in a human pleomorphic glioblastoma cell line (U251 cells), which suggests that the expression of VE-statin/Egfl7 is not specific to endothelial cells and that VE-statin/Egfl7 can be produced in glioma cells. However, the role of VE-statin/Egfl7 and its potential mechanisms in glioma remain unclear. Angiogenesis in such cancers is typically investigated using animal models. Although these models may mimic the *in vivo* physiological status, multiple factors and their environment influence receptors. Thus, it is difficult to investigate the interaction between cancer cells and endothelial cells (two cell types crucial for angiogenesis in glioma) and the potential factors involved. In the present study, Transwell culture plates were employed to construct a co-culture system of U251 cells and HUVECs, which was beneficial for the investigation of the interaction between glioma cells and endothelial cells and the influence of the microenvironment on this interaction *in vitro*. This system was also helpful for investigating the role of VE-statin/Egfl7 in glioma. In our previous study, an siRNA with a short hairpin structure of the VE-statin/Egfl7 gene was introduced into a lentivirus to construct a

Table 2. Effect of VE-statin/Egfl7 siRNA on the adhesion and migration of HUVECs co-cultured with U251 cells (OD, $\bar{x}$±S)

Group	*n*	Adhesion	Migration
		30 min	24 h
CC	5	0.850±0.033*	0.733±0.024
NC	5	0.823±0.036*	0.725±0.041
KD	5	0.406±0.027	0.679±0.030

Note: *vs. KD group, q=30.816, 28.942, P<0.01.

vector expressing a GFP-conjugated siRNA targeting VE-statin/Egfl7. The results showed that this vector could significantly inhibit VE-statin/Egfl7 expression in HUVECs and U251 cells [5, 6]. Based on these findings, the present study aimed to investigate the role of VE-statin/Egfl7 and its mechanism in the endothelium-induced angiogenesis of malignant glioma. Our results revealed that HUVEC growth temporarily slowed and rapidly returned to normal following VE-statin/Egfl7 silencing. In contrast, the migration of endothelial cells was not influenced, although the capability of the cells to adhere was markedly inhibited after silencing the VE-statin/Egfl7 gene. Furthermore, the detection of lumen formation revealed that the endothelial cells failed to form a capillary-like lumen after VE-statin/Egfl7 silencing. These findings suggest that VE-statin/Egfl7 plays an important role in lumen formation during the angiogenesis of glioma by regulating the adherence of endothelial cells.

VE-statin/Egfl7 is a new vasoactive factor that is expressed in early embryonic stages. In normal adult tissues, VE-statin/Egfl7 expression is down regulated or absent but is at a high level in tissues rich in blood vessels, including the lung, heart, and uterus. VE-statin/Egfl7 is reactivated under physiological or pathological conditions and is involved in angiogenesis [7], suggesting that VE-statin/Egfl7 might be reactivated in certain blood vessel-dependent malignancies. Thus, anti-angiogenesis therapy may be promising in the treatment of cancer. Accordingly, over the past 3 years, the correlation between VE-statin/Egfl7 and the occurrence and development of cancer has attracted much attention. Studies have shown that VE-statin/Egfl7 is expressed in multiple malignancies in humans, including glioma. Moreover, VE-statin/Egfl7 may promote the invasion and metastasis of cancer, and its expression is generally associated with poor prognosis [8-10]. Since the proposal of the blood vessel-dependent theory of cancer growth by Folkman, studies have demonstrated that angiogenesis plays important roles in the rapid growth, invasion, and metastasis of cancer cells. Indeed, angiogenesis is a prerequisite for and the basis of the invasion and metastasis of cancer cells, which has been confirmed in numerous studies and is widely accepted. In the present study, we found that VE-statin/Egfl7 can regulate the angiogenic capability of glioma and plays an important in the malignant development of glioma. Further studies should elucidate the significance and mechanism of high VE-statin/Egfl7 expression in malignant human glioma. Although no study has been conducted to date to investigate the molecular mechanism underlying the role of the malignancy of human glioma, there is evidence showing that VE-statin/Egfl7 may activate EGFR to increase the phosphorylation of focal adhesion kinase (FAK), which promotes liver cancer cell migration and metastasis. In Drosophila, VE-statin/Egfl7 may form complexes with the integrin ligand talin to regulate the activity of the integrin αPS2βPS [11], and the binding of talin to an integrin may activate the FAK signaling pathway. In humans, VE-statin/Egfl7 in the extracellular matrix binds to the integrin αvβ3, which is involved in angiogenesis [12]. During embryonic vascular development and the repair of vascular injury, VE-statin/Egfl7 mainly functions to induce the formation of lumen-like structures, which may be attributed to the clustering migration and spatiotemporal arrangement of vascular endothelial cells [13]. In the present study, the angiogenic capability of HUVECs in the presence of U251 cells (a malignant glioma cell line) was significantly inhibited after VE-statin/Egfl7 gene silencing, which was accompanied by a significant change in HUVEC adherence. This change is one of the mechanisms underlying the regulation of angiogenesis in glioma by VE-statin/Egfl7.

The migration of endothelial cells is a crucial step in physiological and pathological angiogenesis. However, we found that the migration of endothelial cells remained unchanged after silencing of the VE-statin/Egfl7 gene. This result might be related to the regulation of the clustering migration of endothelial cells (vascular sprouting) but not to the migration of single cells. Indeed, the clustering migration of cells is

generally present in multicellular organs, whereas single cells migrate independently [13]. The clustering migration of endothelial cells is an important step in angiogenesis and is attributed to the stimulation of linear endothelial cells by vascular growth factors and the subsequent migration of newly generated endothelial cells toward adjacent cells. Schmidt et al. [14] found that VE-statin/Egfl7 had no influence on the migration of single cells but could affect the clustering migration of endothelial cells by transmitting location information to adjacent cells. When VE-statin/Egfl7 expression is deficient, the newly generated endothelial cells adhere to the basement side of the surrounding endothelial cells, resulting in the abnormal accumulation of endothelial cells, but do not arrange along with adjacent cells in a sprouting and linear manner. Thus, the endothelial cells may not migrate forward. One of the mechanisms underlying the effect of VE-statin/Egfl7 is related to the clustering migration of endothelial cells, which precisely regulates the spatiotemporal arrangement of vascular endothelial cells and ensures the accurate migration of endothelial cells and the subsequent formation of capillary lumen-like structures.

The adherence to and integration of endothelial cells into the extracellular matrix are important steps in angiogenesis; otherwise, endothelial cells fail to function normally and become apoptotic. In the present study, silencing of the VE-statin/Egfl7 gene reduced the adherent capability of HUVECs. Parker et al. [15] reported that VE-statin/Egfl7 could promote the adherence of endothelial cells, although the effect was weaker than the effect of matrix proteins. Therefore, the adherence of endothelial cells is facilitated in the presence of VE-statin/Egfl7 alone. Additionally, Schmidt et al. [14] found that VE-statin/Egfl7 could integrate into the extracellular matrix surrounding newly generated blood vessels and form extracellular matrix complexes with fibronectin. However, the adherent capability of VE-statin/Egfl7 is weaker than the capability of other components of the extracellular matrix. Thus, VE-statin/Egfl7 might mainly function to precisely regulate the spatiotemporal arrangement of vascular endothelial cells in the formation of lumen-like structures.

The activation and proliferation of endothelial cells are prerequisites for angiogenesis, although a previous study showed that VE-statin/Egfl7 had no influence on the proliferation of endothelial cells [14]. In the present study, the proliferation of HUVECs was temporarily reduced after co-culture with U251 cells, which was previously attributed to the inhibited proliferation of U251 cells after transfection with VE-statin/Egfl7 siRNA [6]. The inhibition of cancer cell growth may induce the down regulation of the expression of pro-angiogenic factors, which indirectly results in a reduction in HUVEC proliferation. However, prolonged co-culture with U251 cells resulted in an increase in the production of related factors due to the feedback of intracellular signaling pathways, which restored HUVEC proliferation. Thus, VE-statin/Egfl7 may not directly regulate endothelial cell proliferation.

Taken together, our results show that VE-statin/Egfl7 siRNA could inhibit VE-statin/Egfl7 expression in U251 cells and HUVECs and significantly compromise the U251 cell-induced angiogenic capability of HUVECs. These findings were attributed to the clustering migration of endothelial cells via regulation of endothelial cell adherence, which plays an important role in lumen formation. Anti-angiogenesis therapy targeting VE-statin/Egfl7 and gene therapy may therefore be promising for the treatment of glioma.

Acknowledgements

The study was supported by Excellent Youth Project of Bureau of Education of Hunan Province (NO. 11B100) and Scientific Fund of Bureau of Health of Hunan Province (NO. B2010-134), “Western Light” Visiting Scholar Fund Central Ministry of Organization, Scientific Fund of Bureau of Science of Hunan Province (NO. 2009JT3029\14JJ2019) and National Natural Science Foundation of China (81272798).

Disclosure of conflict of interest

None.

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