European Review for Medical and Pharmacological Sciences 2017; 21: 959-972

Glioma cells promote angiogenesis through the release of exosomes containing long non-coding RNA POU3F3

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Abstract. – OBJECTIVE: Angiogenesis is a key event in the progression of gliomas, and emerging evidence suggests that exosomes are signaling extracellular organelles that modulate the tumor microenvironment and promote angiogenesis and tumor progression. This study aimed to explore the mechanism by which glioma-derived exosomes affect angiogenesis.

MATERIALS AND METHODS: qRT-PCR was used to determine the expression level of linc-POU3F3 in glioma tissue as well as glioma cell lines. Ultrafiltration combined with a purification method was used to isolate exosomes derived from A172 cells (A172-Exo) and linc-POU3F3 shRNA-treated A172 cells (shA172-Exo). Transmission electron microscopy, Western blot and tunable resistive pulse sensing (TRPS) were used to identify exosomes. In vitro migration, proliferation, and tube formation experiments, as well as *in vivo* CAM assays, were used to analyze the pro-angiogenesis ability of exosomes. qRT-PCR and Western blot were used to identify expression levels of angiogenesis-related genes and proteins in human brain microvascular endothelial cells (HBMECs) after being cultured with exosomes.

RESULTS: The levels of linc-POU3F3 were upregulated in glioma tissue and significantly correlated with the advanced tumor stage. A172 cells exhibited the highest expression level. A172-Exo was similar to shA172-Exo (50-100 nm in diameter) and expressed Alix, Tsg101 and CD9, while the expression level of linc-POU3F3 in A172-Exo was significantly higher than that in shA172-Exo. HBMECs rapidly internalized A172-Exo and shA172-Exo, and the linc-POU3F3 expression level in HBMECs treated with A172-Exo was significantly higher than the level in HBMECs treated with shA172-Exo. A172-Exo exhibited better function in promoting HBMECs migration, proliferation, tubular-like structure formation *in vitro* and arteriole formation *in vivo.* The gene and protein expression level of bFGF, bFGFR, VEGFA, and Angio in HBMECs treated with A172-Exo was much higher than that of HBMECs treated with shA172-Exo.

CONCLUSIONS: These results indicated that gliomas can induce angiogenesis by secreting exosomes enriched in linc-POU3F3. Exosomes and lncRNA-POU3F3 may, therefore, function as a putative therapeutic target in glioma.

Key Words:
Exosomes, Linc-POU3F3, Glioma cells, Angiogenesis.

Abbreviations

Angio: Angiogenin; ATCC: American Type Culture Collection; CAM: Chick chorioallantoic membrane; CCK8: Cell Counting Kit-8; DMEM: Dulbecco's modified Eagle's medium; EVs: Extracellular Vesicles; FBS: Fetal Bovine Serum; bFGF: basic fibroblast growth factor 2; bFGFR: bFGF receptor; HBMECs: Human brain microvascular endothelial cells; ICAM-1: intercellular adhesion molecule-1; KDR: VEGF receptor 2; LincRNAs: Long intergenic noncoding RNAs; LncRNAs: Long noncoding RNAs; MALAT1: Metastasis-associated lung adenocarcinoma transcript 1; MVBs: Multivesicular bodies; qRT-PCR: Quantitative Real-time polymerase chain reaction; TEM: Transmission electron microscopy; TGFB1: Transforming growth factor beta 1; TRPS: Tunable Resistive Pulse Sensing; VEGFA: Vascular endothelial growth factor A.

Introduction

Highly proliferative tumor cells are in continuous need of oxygen and nutrients. To meet these needs, tumor cells produce large amoun-

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ts of proangiogenic factors that cause abnormal microenvironments and stimulate angiogenesis[1]. Several studies[2,3] have estimated that tumor-associated endothelial cells proliferate 30-40 times faster relative to endothelial cells found in healthy vasculature. Gliomas are the most common and deadly adult primary brain tumors[4], and angiogenesis is also a key event in the progression of gliomas[5]. Studies[6] have demonstrated that vascular density in high-grade glioma is markedly higher than that in low-grade tumors, and glioblastomas are acknowledged to be the most vascularized tumors with the worst prognosis. Therefore, there is a critical need to elucidate potential mechanisms and strategies to inhibit angiogenesis of endothelial cells in and around a glioma for the purpose of developing new anti-angiogenesis therapeutics for gliomas.

Tumor cells are well known to possess the ability to communicate with the different compartments of the tumor microenvironment and ultimately affect neighboring cells. It has been shown that tumor cells can transfer their contents including RNAs and proteins to different types of recipient cells by secreting extracellular vesicles (EVs)[7]. Exosomes are one kind of EVs with a diameter of 40 to 100 nm that are formed in multivesicular bodies (MVBs) by invagination of the endosomal membrane and released into the extracellular space when MVBs fuse with the plasma membrane[8]. Studies[9] have demonstrated that exosomes secreted from tumor cells are involved in cancer growth, angiogenesis, cancer invasion and metastasis, and tumor immunity. For gliomas, evidence has suggested that glioma cells can secrete exosomes into their surrounding microenvironment and modify recipient cells via the transfer of cell-transforming proteins, mRNAs, and specific types of miRNAs[10,11]. For example, Skog et al[11] reported that glioma cell-derived exosomes contained mRNAs, miRNAs and angiogenic proteins, which can be taken up by brain microvascular endothelial cells and stimulate tubule formation and angiogenesis. Nevertheless, the precise mechanism has not been fully elucidated. Long noncoding RNAs (lncRNAs) are nonprotein coding transcripts longer than 200 nucleotides that regulate gene expression at epigenetic transcriptional and post-transcriptional levels[12]. As a subtype of lncRNAs, long intergenic noncoding RNAs (lincRNAs) have been demonstrated to be transcript units located within genomic intervals between two protein coding genes[13]. Increasing evidence has indicated that abnormal expression of lincRNAs plays a critical role in tumor biology, including tumor initiation, progression, and metastasis[14-17]. Our previous research has demonstrated that lincRNA-POU3F3 is overexpressed in gliomas and extraordinarily associated with the tumor WHO grade. Additionally, overexpression of linc-POU3F3 promotes glioma cell viability and proliferation, whereas knockdown of linc-POU3F3 results in the opposite effect[18]. A previous study[19] demonstrated that exosomes also contain various lncRNAs, and Conigliaro et al[20] showed that exosomes released by CD90+ cancer cells are enriched in lncRNA H19, which can be taken up by endothelial cells, promoting their angiogenic phenotype and cell-to-cell adhesion. Thus, we wondered if glioma cells released exosomes enriched in linc-POU3F3 that could be taken up by endothelial cells to promote their angiogenesis.

In the present study, we osserved that exosomes released by glioma cell lines A172 (A172-Exo) contained linc-POU3F3, could be internalized by endothelial cells, and, in turn, were able to influence endothelial cells by stimulating angiogenesis-related gene and protein expression, finally promoting angiogenesis. These findings shed new light on glioma progression and indicate that exosomes could be used as a putative therapeutic target in gliomas.

Materials and Methods

Ethics Statement

The protocols employed in this work and the use of human tissues were approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University and conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki and the local legislation. All patients were informed and provided consent to use excess pathological specimens for research purposes. Also, all experimental protocols were carried out in accordance with the relevant guidelines and regulations.

Patients and Tissue Samples

Patients with glioma (n = 82) who underwent an initial surgery in the Second Affiliated Hospital of Nanchang University were retrospectively selected for this study. No patients had received therapy before resection. All tumors were classified on the basis of the WHO criteria for tumors of the central nervous system. The clinical characteristics of all patients are summarized in Table I. Glioma tissue and adjacent normal tissue were immediately frozen in liquid nitrogen and stored at -80°C until further use.

Cell Lines and Culture Conditions

Human glioma cell lines (A172, U87-MG, U251, T98G) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA); all glioma cell lines and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% (vol/vol) fetal bovine serum (FBS, Gibco). Human brain microvascular endothelial cells (HBMECs) were purchased from 3H Biomedical (Stockholm, Sweden) and cultured in EC medium supplemented with 5% FBS and EC growth supplement. HBMECs at passages 2-10 were used in the experiments as described below. The routine culture was done in a humidified incubator maintained at 37°C with 5% CO_2 and 95% air.

shRNA Transfection

To obtain the shPOU3F3-expressing A172 cells, the pGV248-POU3F3 shRNA and scramble shRNA obtained from Genepharma (Shanghai, China) were transfected into 293T cells along with the packaging plasmids. The lentivirus partials were harvested, and the knockdown efficiency was determined by qRT-PCR after co-transfection for 48 h. The lentiviruses with pGV248-POU3F3 shRNA or scramble shRNA were used to infect A172 glioma cells to construct stable expression cell lines for the experiments below.

Exosome Isolation and Purification

A172 glioma cells were grown with 10% depleted FBS (FBS was pre-depleted of bovine exosomes by ultracentrifugation at 4°C, 100.000 g, 16 h). When cells were confluent to 70%, they were rinsed three times with PBS and cultured for 48 h with 10% depleted FBS. Exosomes were isolated and purified from A172 cell supernatant as previously described[21]. Exosomes derived from untreated A172 cells were termed A172-Exo, and exosomes derived from lincRNA-POU3F3 shRNA-treated A172 cells were termed shA172-Exo. An equal volume of medium without culturing cells was obtained using the same method for the A172 exosomes and was referred to as the "control medium". The exosome protein content was determined by using the bicinchoninic acid assay (Thermo Fisher, Waltham, MA, USA) as previously described[22]. The bovine serum albumin (BSA) ranged from 2 mg/mL to 25 µg/mL to generate a calibration curve. The absorbance was read at 562 nm using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

Exosome Morphology and Size Analysis

Transmission electron microscopy (TEM) was used to identify the morphology of exosomes as previously described[21]. A172 exosomes were visualized on a Hitachi H-7650 transmission electron microscope (Hitachi, Tokyo, Japan), and images were captured by a digital camera (Olympus, Tokyo, Japan). Nanoparticle tracking analysis (NTA) measurements were performed using tunable resistive pulse sensing (TRPS, IZON qNano, Christchurch, New Zealand) technology to investigate the size and numbers of exosomes, as previously reported[23].

Table I. Association between lncRNA-POU3F3 expression and clinicopathological features of glioma patients.

		Relative POU3F3 expression		
Characteristic	**No. of patients (%)**	**High**	**Low**	***p*-value***
Sex				
Male	42 (51.22%)	25 (30.49%)	17 (20.73%)	0.529
Female	40 (48.78%)	21 (25.61%)	19 (23.17%)	
Age				
< 50 (39.46 ± 8.27)	44 (53.66%)	21 (25.61%)	23 (28.05%)	0.346
≥ 50 (57.89 ± 4.77)	38 (46.34%)	22 (26.83%)	16 (19.51%)	
Tumor size (cm)				
< 5	50 (60.98%)	28 (34.15%)	22 (26.83%)	0.127
≥ 5	32 (39.02%)	20 (24.39%)	12 (14.63%)	
WHO grade				
I/II	34 (41.46%)	11 (13.41%)	23 (28.05%)	0.009

**p*-values were determined using a 2-sided chi-square test or a 1-way analysis of variance.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Assay

RNAzol RT (Molecular Research Center, USA) was used to extract total RNA from glioma tissue, adjacent normal tissue, glioma cell lines, HBMECs and exosomal sources[24]. qRT-PCR for human-specific repeat sequences was performed as previously described[21]. To identify the gene expression of HBMECs after treatment with A172-Exo and shA172-Exo, 8 × 10^5 HBMECs were seeded onto six-well plates and cultured with fresh medium containing 100 µg/mL exosomes for 48 h. The primers were obtained from GenePharma (Shanghai, China), and the following human primers were used: linc-POU3F3, basic fibroblast growth factor (bFGF), bFGF receptor (bFGFR), vascular endothelial growth factor A (VEGFA), VEGF receptor 2 (KDR), transforming growth factor beta 1 (TGFB1), intercellular adhesion molecule-1 (ICAM-1), and angiogenin (Angio). GAPDH was used to normalize lincRNA in the tissue, cells and exosomes. The primer sequences used in this study are summarized in Table II. Each sample was analyzed in triplicate for yield validation. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative quantification of gene expression levels.

Protein Extraction and Western Blotting

Western blotting was used to identify A172 exosome markers Alix, Tsg101, and CD9[25] and angiogenesis-related protein expression in HBMECs. To identify the protein expression in HBMECs after treatment with A172-Exo and shA172-Exo, 8 × 10^5 HBMECs were seeded onto six-well plates and cultured with fresh medium containing 100 µg/mL exosomes for 48 h. Later, the cells and exosome pellets were lysed in lysis buffer (Roche Diagnostics, Mannheim, Germany); then, 5 × protein loading buffer was added directly to the protein sample and heated at 95°C for 5 min. Next, equal amounts of protein were loaded and separated on SDS-PAGE polyacrylamide gels, and proteins were blotted to a nitrocellulose membrane (Whatman, Maidstone, Kent, UK). The primary antibodies used were Alix (mouse monoclonal anti-Alix, 1:1000, Abcam, Cambridge, UK), Tsg101 (mouse monoclonal anti-Tsg101, 1:200, Abcam), CD9 (rabbit monoclonal anti-CD9, 1:1000, Abcam), VEGFA (mouse monoclonal anti- VEGFA, 1:100, Abcam), bFGF (rabbit monoclonal anti-bFGF, 1:1000, Abcam), bFGFR (mouse monoclonal anti-bFGFR, 1:500, Abcam), Angio (mouse monoclonal anti-Angio, 1:250, Abcam), and GAPDH (mouse monoclonal anti-GAPDH, 1:5000, Abcam). The primary antibodies were incubated overnight at 4°C followed by washing and the application of the HRP-conjugated goat anti-rabbit secondary antibody (1:2000, Thermo Fisher, Waltham, MA, USA) and goat anti-mouse secondary antibody (1:2000, Thermo Fisher). Proteins were detected by using enhanced chemiluminescence (Thermo Fisher, Waltham, MA, USA) and imaged using a Molecular Imager VersaDoc 4000 system (Bio-Rad, Hercules, CA, USA).

Uptake of Exosomes by HBMECs

Immunofluorescence staining was performed to confirm that HBMECs take up the exosomes. A172 cells were labeled with 3,3'-dihexadecyloxacarbocyanine perchlorate (CM-DiO, Invitrogen, Carlsbad, CA, USA) according to the supplier's instructions. Exosomes derived from DiO-labeled A172 cells were routinely collected, and then HBMECs were incubated with 100 µg/ml exosomes for 2 h and 12 h. Next, HBMECs were rinsed with PBS and fixed with 4% paraformaldehyde at room temperature for 30 min. Then, HBMECs were pre-incubated with sodium borohydride (1

Table II. Primers used for quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR).

Genes	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
h- linc-POU3F3	AATCACTGCAATTGAAGGAAAAA	CCTTGTTTTCCAACCCTTAGACT
h- bFGF	CAATTCCCATGTGCTGTGAC	ACCTTGACCTCTCAGCCTCA
h- bFGFR	GACGGCTCCTACCTCAA	GCTGTAGCCCATGGTGTTG
h- VEGFA	CGCTCGGTGCTGGAATTTGA	AGTGGGGAATGGCAAGCAAA
h- KDR	GTGATCGGAAATGACACTGGAG	CATGTTGGTCACTAACAGAAGCA
h- TGFB1	TTGAGGGCTTTCGCCTTAGC	TGAACCCTGCGTTGATGTCC
h- ICAM-1	AACCCATTGCCCGAGC	GGTGAGGATTGCATTAGGTC
h- Angio	CTCGCTTCGGCAGCACA	GGTGGTCGGAGATTCGTAGC
h- GAPDH	ATCCCATCACCATCTTCC	GAGTCCTTCCACGATACCA

mg/mL in PBS) to reduce autofluorescence, later incubated overnight at 4°C with primary antibody CD31 (mouse monoclonal anti-CD31, 1:100; Abcam), and incubated for 1 h with a secondary antibody conjugated to Alexa Fluor 594 (1:200; Abcam). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (0.5 μg/Ml, Invitrogen, Carlsbad, CA, USA). Images were obtained using a fluorescence microscope (Leica, Solms, Germany).

Endothelial Cell Migration Assay

The scratch wound assay was used to analyze the migration ability of HBMECs. Briefly, 2×10^5 cells were seeded into 12-well plates and maintained at 37°C to permit cell adhesion and the formation of a confluent monolayer. Next, these confluent monolayers were 'scratch'-wounded by a p200 pipet tip. The medium was removed and rinsed once with PBS to remove the debris and smooth the edge of the scratch. The medium was then replaced with fresh 1% FBS EC medium containing 100 μg/mL A172-Exo or 100-μg/mL shA172-Exo or control medium. Wound closure was monitored by collecting digital images at 0-h, 8-h, and 16-h intervals after the scratch, and digital images were captured using an inverted microscope (Leica). Wound closure was analyzed using the MetaMorph software, and the wound area at each time point was normalized to its corresponding area at 0 h.

Endothelial Cell Proliferation Assay

A Cell Counting Kit-8 assay (CCK-8; Dojindo, Kumamoto, Japan) was used to assess cell proliferation. Briefly, HBMECs were seeded at 5×10^4 cells/mL (100 μL/well) in a 96-well plate. After quiescence for 12 h, cells were treated with fresh 1% FBS EC medium containing 100 μg/mL A172-Exo or 100 μg/mL shA172-Exo or control medium. On days 0, 1, 2, 3, 4, and 5, 10 μL CCK-8 solution was added to the HBMECs and incubated for 3 h at 37°C. The absorbance was measured at 450 nm using a microplate reader. All of these experiments were performed in triplicate and repeated at least three times.

Endothelial Cell Capillary-Like Tube Formation Assay

In vitro capillary-like tube formation was evaluated on growth factor-reduced Matrigel (BD Biosciences, Sparks, MD, USA). Here, 2×10^4 HBMECs were seeded onto the plated Matrigel with fresh 1% FBS EC medium containing 100 μg/mL A172-Exo or 100 μg/mL shA172-Exo or control medium. Tube formation was quantified at 6 h. The total number of branch points, total tube length, covered area, and total loops per image were measured by a blinded independent observer.

Chick Chorioallantoic Membrane (CAM) Assay

The CAM assay was performed to confirm the effect of exosomes on blood vessel formation in developing fertilized chicken eggs[26,27]. A gelatin sponge with 5 mm diameter carried 200 μg A172-Exo, 200 μg shA172-Exo, or control medium was implanted in CAMs. After the CAMs were incubated for 72 h, they were photographed using a camera (Nikon, Tokyo, Japan). Tertiary arterioles (the number of tertiary vessels divided by the number of branches) were quantified, and the data are presented as the mean ± SE of the total number of tertiary vessels in 3 independent CAMs for each treatment.

Statistical Analysis

Statistical analysis was performed using the SPSS Graduate Pack, version 11.0, statistical software (SPSS Inc., Chicago, IL, USA). Differences between two groups were analyzed by Student's *t*-test. Data were expressed as the means ± standard deviation (SD) of three independent experiments. *p*-values less than 0.05 were considered significant.

Results

The Expression Level of Linc-POU3F3 was Significantly Increased in Glioma Tissue

The expression level of linc-POU3F3 was assessed in 82 paired glioma samples and corresponding adjacent normal tissue samples using qRT-PCR normalized to GAPDH. First, we studied the correlation between linc-POU3F3 expression and clinical pathological features, and, as shown in Table I, linc-POU3F3 expression correlated with the WHO grade but did not correlate with the patients' sex, age, or tumor size. Compared with the corresponding adjacent normal tissue samples, linc-POU3F3 expression in gliomas was increased (fold change ≥ 2) in 45 cases (54.9%), whereas 37 cases (45.1%) showed a decrease or no significant difference. Overall, the expression level in gliomas was significantly higher than that in adjacent normal tissue (Figure 1A and Table I,

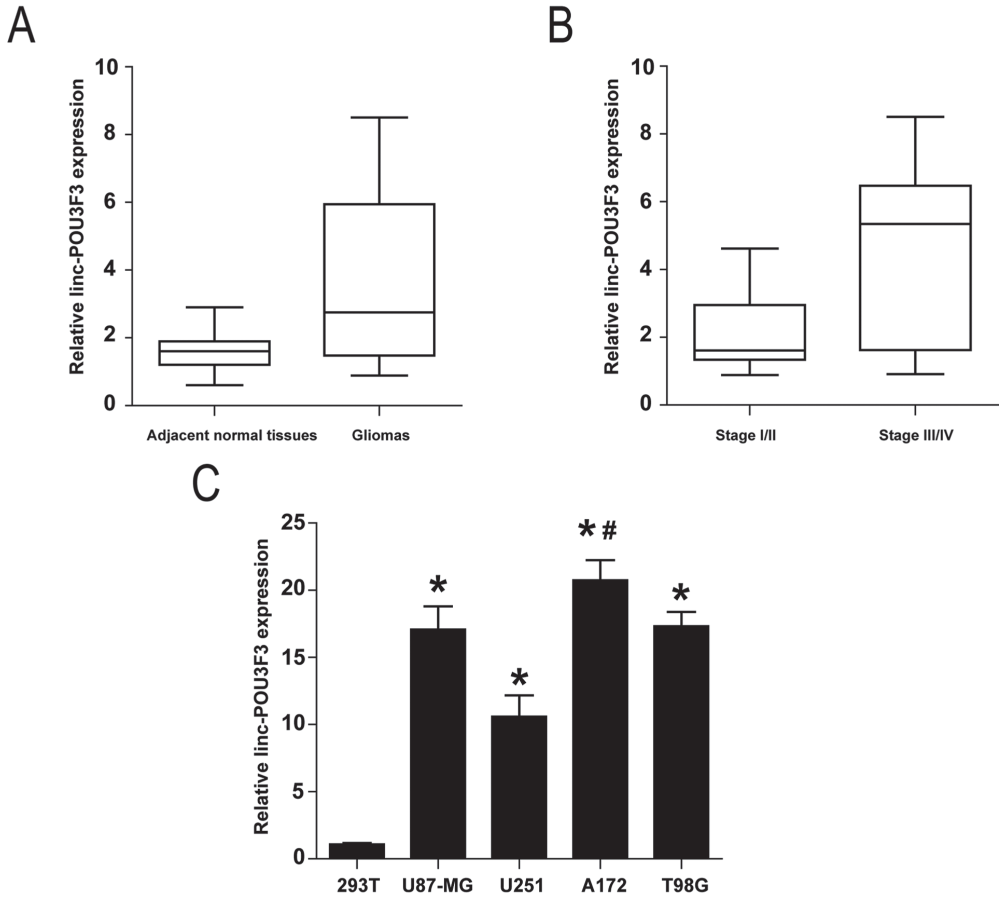

Figure 1. Abnormal linc-POU3F3 expression is associated with glioma. ***(A)*** The relative expression level of linc-POU3F3 in glioma tissue and adjacent normal tissue was measured by qRT-PCR (N = 82). RNA relative expression levels were normalized against the GAPDH transcript expression levels. 45 cases (54.9%) showed increased linc-POU3F3 expression, whereas 37 cases (45.1%) showed a decrease or no significant difference. ***(B)*** 34 cases (49.1%) of the 48 WHO III/IV glioma patients showed high expression of linc-POU3F3 (fold change ≥ 2), whereas 11 cases (17.4%) of the 34 WHO I/II glioma patients showed high expression. A fold change of ≥ 2 was defined as high expression of linc-POU3F3, and the rest of the cases were classified as low linc-POU3F3 expression. ***(C)*** Linc-POU3F3 levels were evaluated by qRT-PCR in four glioma cell lines, and non-glioma 293T cells were used as controls. A172 cells showed the highest expression level of linc-POU3F3 (*compared with 293T cells, $p < 0.01$; $^{\#}$compared with U87-MG, U251, and T98G cells, $p < 0.05$).

$p < 0.05$). Furthermore, as shown in Figure 1B and Table I, the linc-POU3F3 expression level significantly increased with the increasing WHO grade of the gliomas. In WHO III/IV gliomas, there were 34 cases (41.5%) that showed high expression of linc-POU3F3 (fold change ≥ 2), whereas only 11 cases (13.4%) with WHO I/II gliomas showed high expression. The expression level in WHO III/IV gliomas was significantly higher than that in WHO I/II gliomas ($p < 0.01$). We also found that the linc-POU3F3 expression levels in glioma cell lines U87MG, U251, A172, and T98G were all higher than those in non-glioma 293T cells ($p < 0.01$). As shown in Figure 1C, the linc-POU3F3 level was the highest and lowest in the A172 cells (20.75-fold compared to that in the 293T cells) and U251 cells (10.59-fold compared to that in the 293T cells), respectively. This result implied that linc-POU3F3 overexpression might participate in the development of gliomas and might serve as a novel marker for the poor prognosis or progression of a glioma.

Characterization of Exosomes Released by A172 Cells and shA172 Cells

Because the glioma cell line A172 exhibited the highest expression level of linc-POU3F3 among the four cell lines, the A172 cells were used in the following study. Figure 2A shows that linc-POU3F3 was efficiently silenced using shRNAs (shRNA1, shRNA2 and shRNA3) in A172 cells compared with its expression in ne-

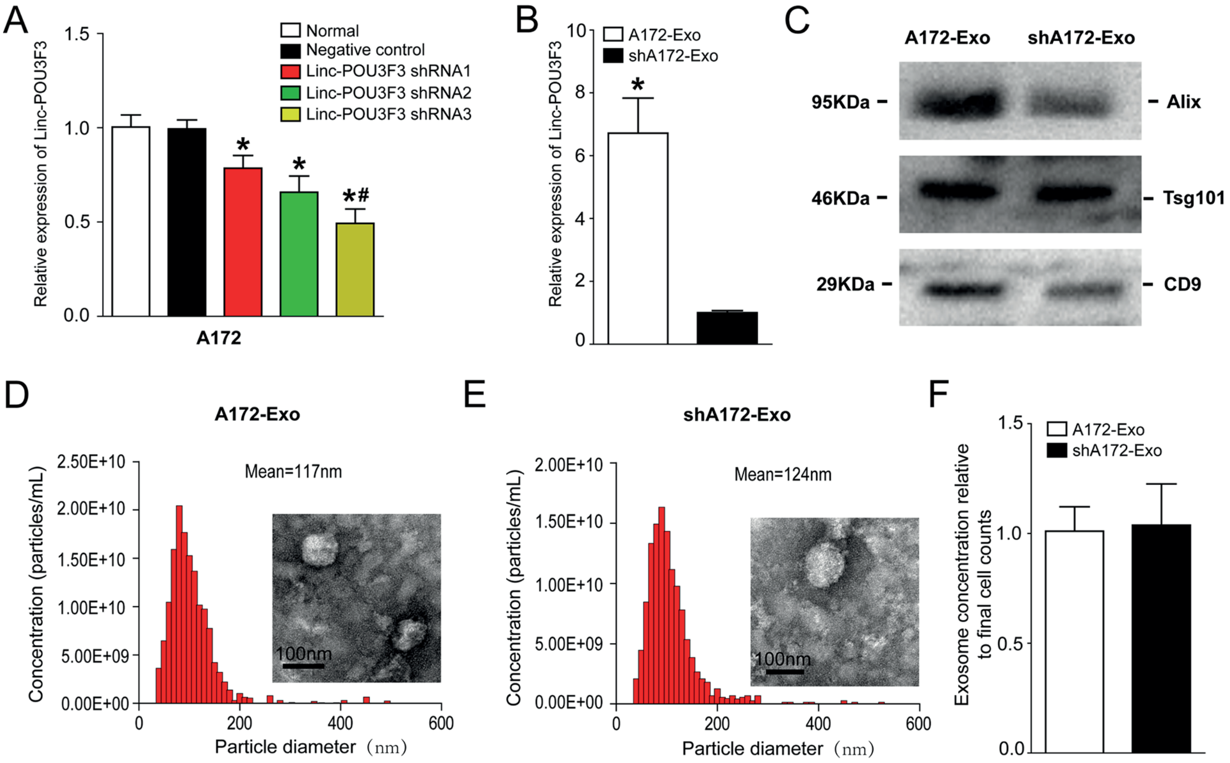

Figure 2. Characterization of exosomes released by A172 cells and shA172 cells. ***(A)*** The knockdown effects of linc-POU3F3 were measured by qRT-PCR in A172 cells transfected with shRNAs or its negative controls; the linc-POU3F3 shRNA3 showed the best function in silencing linc-POU3F3 mRNA (*compared with negative controls, $p < 0.01$; $^{\#}$compared with shRNA1 and shRNA2, $p < 0.05$). ***(B)*** A172-Exo were highly enriched in the linc-POU3F3 transcript compared with its expression in shA172-Exo (*$p < 0.01$). ***(C)*** Western blotting analysis of exosomal markers Alix, Tsg101, and CD9 in A172-Exo and shA172-Exo; equal amounts of exosomes (300 ng) were used for the assay. ***(D-E)*** The nanoparticle size distribution and concentrations for A172-Exo and shA172-Exo were obtained by NTA. The morphology of A172-Exo and shA172-Exo was observed by TEM. ***(F)*** The concentrations of A172-Exo and shA172-Exo were normalized to final cell counts; there were no significant differences observed between A172-Exo and shA172-Exo ($p > 0.05$).

gative controls and untreated cells (normal). The linc-POU3F3 shRNA3 showed the best function in silencing linc-POU3F3 mRNA and was chosen to be used in the following experiments. Furthermore, we identified the characteristics of the exosomes released by A172 cells and linc-POU3F3 shRNA-treated A172 cells (shA172). As shown in Figure 2C, the presence of exosomal markers Alix, Tsg101 and CD9 was confirmed by Western blot in the two kinds of exosomes. The morphology of the exosomes derived from A172 cells (A172-Exo) and shA172 cells (shA172-Exo) was observed by TEM. A172-Exo were similar to shA172-Exo (50-100 nm in diameter), and each of those vesicles showed the classical cup or round-shaped appearance (Figure 2D and E). To further investigate the size distribution profile of the exosomes derived from A172 cells and shA172 cells, we performed NTA using the TRPS system. The nanoparticle size distribution of A172-Exo was similar to that of shA172-Exo, and the peaks in the particle size were ~100 nm within the expected size of exosomes (Figure 2D and E). When the nanoparticle concentrations were normalized to cell numbers at the time of harvest, there were no significant differences observed between A172-Exo and shA172-Exo (Figure 2F). These results indicate that the size and concentration of exosomes secreted from A172 cells was not affected when cells were treated with linc-POU3F3 shRNA. Finally, qRT-PCR was used to identify the expression level of linc-POU3F3 in A172-Exo and shA172-Exo. As shown in Figure 2B, A172-Exo were highly enriched in the linc-POU3F3 transcript compared with shA172-Exo ($p < 0.01$), which indicated that A172 cells released exosomes containing linc-POU3F3, and inhibiting linc-POU3F3 expression in A172 cells decreased the quantity of linc-POU3F3 in the exosomes.

A172-Exo and shA172-Exo Could be Internalized by HBMECs

We posited that exosomes must be internalized by HBMECs to achieve their function. Therefore, we labeled A172-Exo and shA172-Exo with plasma membrane fluorescent dye DiO to examine whether A172-Exo and shA172-Exo could be internalized into HBMECs. As Figure 3A shows, HBMECs rapidly internalized exosomes from both cell types within 2 h, and the fluorescence intensity reached its maximum at 12 h in both groups. Furthermore, we identified the linc-POU3F3 expression level in HBMECs when incubated with 100 µg/mL A172-Exo or shA172-Exo for 24 h. The qRT-PCR results showed that the linc-POU3F3 expression level in HBMECs treated with A172-Exo was significantly higher than that in HBMECs treated with shA172-Exo (Figure 3B, $p < 0.01$). These results suggest a transport of linc-POU3F3 from A172 cells to HBMECs by exosomes, even though we cannot exclude a stimulation of endogenous lincRNA.

A172-Exo and shA172-Exo Regulated HBMEC Migration, Proliferation, and Tube Formation in Vitro and Angiogenesis in Vivo

We further investigated the functional roles of A172-Exo and shA172-Exo in regulating angiogenesis *in vitro* and *in vivo*. First, the cell migration, proliferation, and tube formation capacities were assessed using a series of *in vitro* angiogenesis assays. Strikingly, the scratch wound assay showed that both A172-Exo and shA172-Exo significantly enhanced the motility of HBMECs, while A172-Exo showed a better pro-migratory

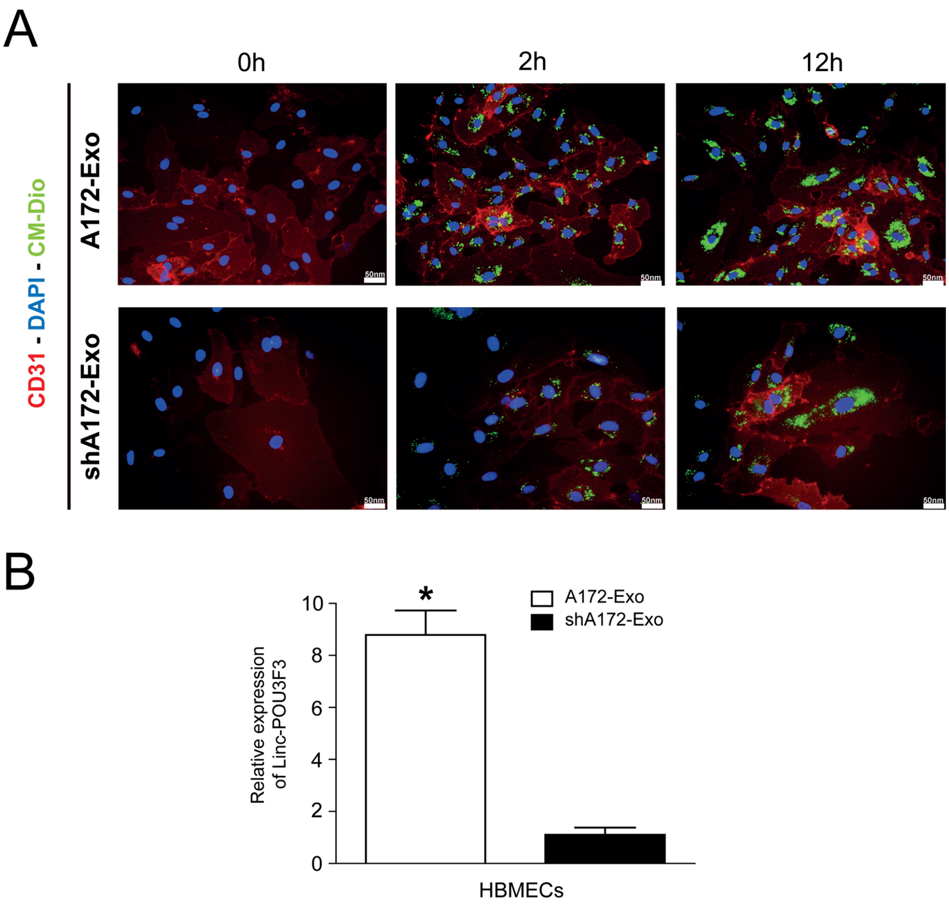

Figure 3. A172-Exo and shA172-Exo could be internalized by HBMECs. ***(A)*** Immunofluorescence images of DAPI (*blue*)-CD31 (*red)* HBMECs co-cultured with CM-DiO (green)-labeled A172-Exo and shA172-Exo. The time points of observation are indicated. HBMECs rapidly internalized A172-Exo and shA172-Exo within 2 h, and the fluorescence intensity reached its maximum at 12 h. ***(B)*** qRT-PCR was used to measure the linc-POU3F3 expression level in HBMECs incubated with 100 µg/mL A172-Exo or shA172-Exo for 24 h; the linc-POU3F3 expression level in HBMECs treated with A172-Exo was significantly higher than that in HBMECs treated with shA172-Exo ($*p < 0.01$).

ability ($p < 0.05$, Figure 4A and B). The CCK-8 cell counting analysis showed that A172-Exo significantly stimulated endothelial cell proliferation, while shA172-Exo only slightly promoted endothelial cell proliferation, when compared to that observed in the control medium group ($p < 0.05$, Figure 4C). To determine their effects on tube formation, the cells were seeded on matrigel and cultured for 6 h. The total branching points, total tube length, tube-covered area, and total loops at the indicated time were measured to quantify the ability of HBMECs to form tubes. Compared with that observed in the control medium group, all indicators that evaluated the capability of endothelial cells to form tubes were increased in HBMECs incubated with A172-Exo and shA172-Exo, indicating that both A172-Exo and shA172-Exo could promote tube formation, and A172-Exo exhibited a stronger pro-tube formation function ($p < 0.05$, Figure 4D-H). Next, we performed the CAM assay to estimate the pro-angiogenesis function of exosomes *in vivo*. As shown in Figure 4I and J, the number of newly formed arterioles correlated well with the effect of the exosomes, and samples treated with A172-Exo clearly showed formation of more new arterioles than samples treated with shA172-Exo ($p < 0.05$), which was similar to the results of our *in vitro* data. These data suggest that exosomes released from glioma cells play positive

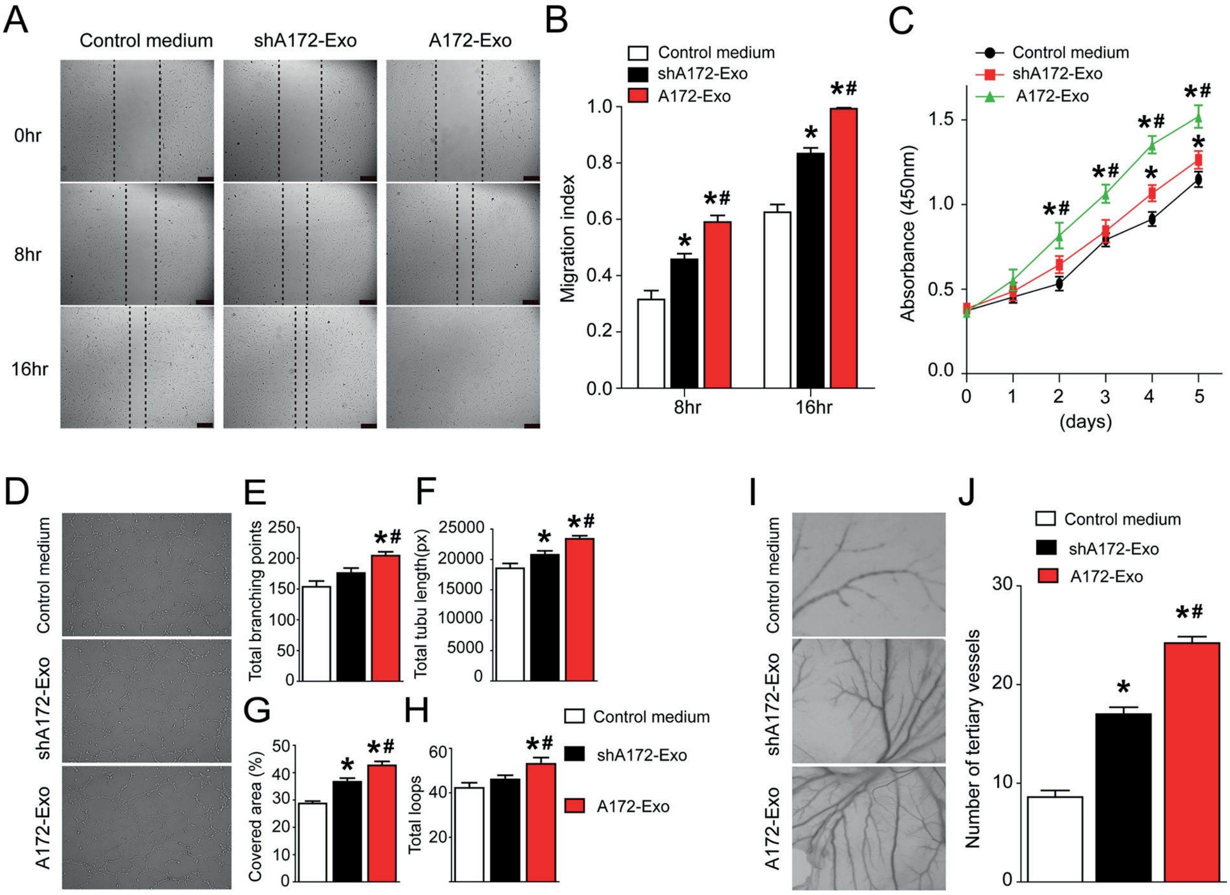

Figure 4. A172-Exo and shA172-Exo regulated HBMEC migration, proliferation, and tube formation *in vitro* and angiogenesis *in vivo*. ***(A-B)*** The migration ability was measured by using the scratch wound assay; both A172-Exo and shA172-Exo significantly enhanced the motility of HBMECs, while A172-Exo showed a better pro-migratory ability. ***(C)*** Proliferation was measured by using the CCK-8 assay; A172-Exo significantly stimulated HBMEC proliferation, while shA172-Exo only slightly promoted HBMEC proliferation. ***(D-H)*** A tube formation test was performed on growth factor-reduced Matrigel. The quantity of total branching points, total tube length, tube-covered area, and total loops at 6 h in HBMECs treated with A172-Exo were higher than that in HBMECs treated with shA172-Exo. ***(I-J)*** Just as in the *in vitro* study, A172-Exo induced CAM arteriole formation to a greater extent than that induced by shA172-Exo. Representative pictures of the CAM assays are presented in the left panels, and the numbers of vessels are in the right panel (n = 3). (*represents $p < 0.05$ when compared to control medium; #represents $p < 0.05$ when compared to shA172-Exo).

roles in angiogenesis, and linc-POU3F3 acts as a possible mediator of pro-angiogenesis properties of exosomes released by glioma cells.

Exosomal linc-POU3F3 Derived from A172 Cells Regulates Angiogenesis-Related Gene and Protein Expression in HBMECs

To investigate the possible mechanisms of linc-POU3F3 in glioma cell-derived exosomes as a mediator of proangiogenic stimuli in HBMECs, we incubated HBMECs with 100 µg/mL A172-Exo or shA172-Exo and used qRT-PCR and Western blot to detect the expression level of angiogenesis-related genes and proteins in HBMECs. As shown in Figure 5A, linc-POU3F3 overexpression in HBMECs (treated with A172-Exo) induced a significant increase in the bFGF, VEGFA, bFGFR, and Angio transcripts compared to that observed in cells treated with shA172-Exo ($p < 0.05$, Figure 5A), while no modulation was observed for the expression of KDR, ICAM, and TGFB1. The Western blot assay (Figure 5B-C) showed a substantial increase in bFGF, VEGFA, bFGFR, and Angio formation in HBMECs incubated with A172-Exo when compared with that expressed in cells treated with shA172-Exo ($p < 0.05$). Taken together, these results demonstrated that A172-Exo promote angiogenesis partly because linc-POU3F3 in A172-Exo increased the expression level of bFGF, VEGFA, bFGFR, and Angio.

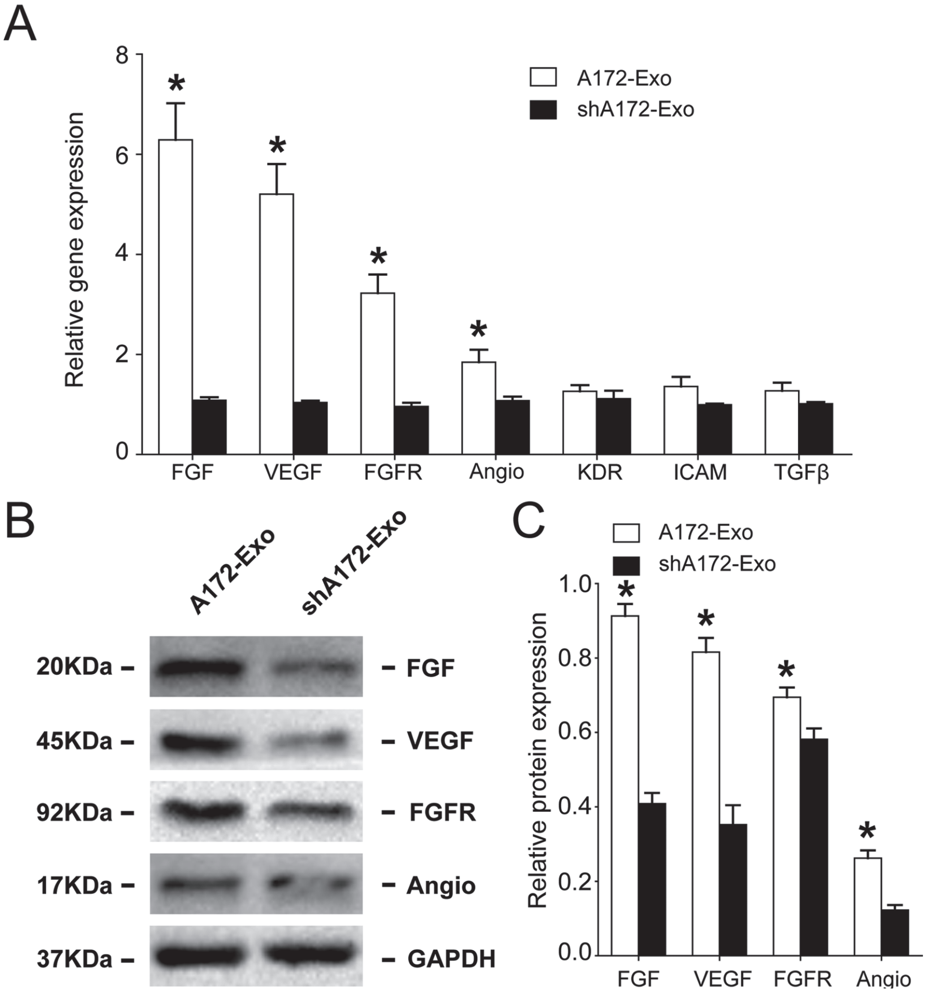

Figure 5. Exosomal linc-POU3F3 derived from A172 cells regulates angiogenesis-related gene and protein expression in HBMECs. ***(A)*** qRT-PCR analysis of the expression level of angiogenesis-related genes in HBMECs treated with A172-Exo or shA172-Exo. Compared with that observed in the HBMECs treated with shA172-Exo, A172-Exo significantly upregulated bFGF, VEGFA, bFGFR, and Angio gene expression. ***(B)*** Western blot analysis of the expression level of angiogenesis-related proteins in HBMECs treated with A172-Exo or shA172-Exo. Compared with that observed in the HBMECs treated with shA172-Exo, A172-Exo significantly increased bFGF, VEGFA, bFGFR, and Angio protein expression. $*p < 0.05$.

Discussion

Despite advances in surgical and medical therapy, glioma consistently remains a fatal disease[28]. Currently, the formation of abnormal tumor vasculature is believed to be one of the major reasons for glioma development, but the mechanism of tumor vascularization is complex and still unclear[29]. Tumor-derived exosomes can act as mediators in the intercellular communication of the tumor microenvironment, constructing a fertile environment to support tumor proliferation, angiogenesis, and invasion and prepare a premetastatic niche through the intercellular transfer of proteins, mRNAs, miRNAs, and lncRNAs[30,31]. Therefore, we conducted the present work to clarify the possible relationships between gliomas and angiogenesis and to explore the potential application of glioma-derived exosomes in the diagnosis and treatment of glioma. As a newly discovered class of non-coding genes, lncRNAs have been recently found to be pervasively transcribed in the genome. Emerging biochemical evidence has revealed that lncRNAs have an incredible function in the control of gene expression and chromatin structure. For example, lncRNAs can recruit chromatin-modifying proteins, modulate protein-DNA binding, organize nuclear architecture, regulate mRNA stability and translation, modulate mRNA levels by competing for microRNA binding and directly alter protein localization and function[32-36]. Genome-wide profiling studies have revealed that lncRNA expression profiles between normal brain tissue and gliomas are significantly different. Certain lncRNAs are involved in cancer progression, and gliomas of different malignancy grades have also been shown to have differential lncRNA expression[37-40]. For example, Ma et al[38] has shown that lncRNA MALAT1 expression is increased in glioma tissue and significantly associated with the WHO grade. Additionally, increased lncRNA MALAT1 expression was a poor independent prognostic predictor for glioma patients. Our previous research demonstrated that linc-POU3F3 is overexpressed in glioma tissue compared with expression in the adjacent normal tissue and positively correlates with the tumor WHO grade; overexpression of linc-POU3F3 promotes glioma cell viability and proliferation, whereas knockdown of linc-POU3F3 shows the opposite effect[18]. However, the exact mechanism for how linc-POU3F3 affects glioma progression is still unclear and needs to be further explored. Most solid tumors have been recognized to rely on angiogenesis for continuous growth. Tumor blood vessels provide nutrition and oxygen to the tumor, resulting in tumor progression[41]. Similarly, angiogenesis is also a key event in the progression of gliomas. Studies[5,6] have demonstrated that the vascular density in high-grade gliomas is markedly higher than that in low-grade tumors, and glioblastomas are acknowledged to be the most vascularized tumors with the worst prognosis. Tumor cells under metabolic/hypoxic stress undergo an angiogenic switch that results in increased expression and release of angiostimulatory growth factors, such as FGFs, VEGF, and Angio, which bind to their receptors on the endothelial cell surface, resulting in activation of the angiogenesis cascade[42]. Several investigations[43,44] have described exosomes as signaling extracellular organelles that modulate the tumor microenvironment, promoting angiogenesis and tumor progression. Huang et al[45] have found that exosomes derived from hypoxic colorectal cancer cells can promote endothelial cell proliferation and migration, as well as tumor growth and angiogenesis. Also, Conigliaro et al[20] observed that exosomes released by CD90+ cancer cells can modulate endothelial cells and promote an angiogenic phenotype and cell-to-cell adhesion. Also, lncRNA profiling revealed that exosomes from CD90+ cells are enriched in lncRNA H19, which can be taken up by endothelial cells and regulate their function. Based on these findings, we hypothesized gliomas promoted angiogenesis by packaging linc-POU3F3 into exosomes and transferring them to endothelial cells to regulate their angiogenesis function.

In the present study, firstly, we determined the expression level of linc-POU3F3 in 4 glioma cell lines and found that A172 glioma cells expressed the highest level of linc-POU3F3. Secondly, we applied shRNA transfection technology to inhibit linc-POU3F3 expression in A172 cells. We showed that silencing linc-POU3F3 in A172 cells decreased its expression level in exosomes but did not influence the size or concentration of the exosomes. Thirdly, we investigated the function of A172-Exo and shA172-Exo in regulating HBMEC angiogenesis. We found that both A172-Exo and shA172-Exo were effectively internalized by HBMECs, but A172-Exo exhibited a better ability to promote HBMEC migration, proliferation, and tubular-like structure formation *in vitro* and arteriole formation *in vivo*. At last, we demonstrated that A172-Exo had a better pro-angiogenesis function partly from improving the linc-POU3F3 expression level in HBMECs. Linc-POU3F3 overexpression in HBMECs was able to upregulate the gene and protein expression of bFGF, VEGFA, bFGFR, and Angio, which have been proven to be key pro-angiogenesis factors in the regulation of angiogenesis. Taken together, these results suggested that glioma cells regulated angiogenesis partly because they were able to package linc-POU3F3 inside exosomes, thus delivering it to endothelial cells and influencing them in a pro-angiogenesis manner by upregulating angiogenesis-related gene and protein expression.

Conclusions

The present work demonstrated that glioma-derived exosomes transfer linc-POU3F3 as a key mediator of non-contact cell-to-cell communication and the regulation of glioma angiogenesis. In light of several publications highlighting the importance of exosomes in cancer biology and the results described here, we suggest that targeting exosomes and linc-POU3F3 might represent two new therapeutic applications in glioma treatment and recommend that they be further explored for future clinical use.

Author's Contributions

GHX and LW conceived the study, designed the experiments, and provided their funds to the study; GWH and HLL participated in the cell culture, shRNA infection and article writing; YC and YL were responsible for qRT-PCR and Western blot. WT and YML were responsible for CAM assays and performed the statistical analysis of all experimental data. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by the National Scientific Foundation of China grant (No. 81560411, No. 81560193). The authors have no financial relationship with the organization that sponsored the research or any other conflict of interest regarding this research.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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