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Original Article

Expression of EGFL7 gene in neural stem cells and vascular endothelial cells and its effect and mechanism

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Abstract: Objective: We performed co-culture and RNA interference to determine the role played by epidermal growth factor-like domain 7 (EGFL7) in the angiogenesis induced by neural stem cells and proposed the mechanism tentatively. Method: NSCs-HUVECs co-culture system was established using Transwell technique, and the interaction between the neural stem cells and the endothelial cells was simulated in vitro. The siRNA lentiviral vector targeting EGFL7 gene was constructed and transfected into the NSCs-HUVECs system. Thus the effect of EGFL7 gene on angiogenesis of the vascular endothelial cells (VECs) was discussed by proliferation, adhesion and tube formation tests of VECs. Results: After the silencing of EGFL7 gene, the EGFL7 expression level in HUVECs decreased and the adhesion capacity was inhibited significantly. Tube formation test indicated that no tubes were formed by the VECs after silencing of EGFL7 gene. Conclusion: EGFL7 played a key regulatory role in angiogenesis induced by neural stem cells by regulating the adhesion capacity of VECs.

Keywords: EGF-like domain containing protein 7, angiogenesis, RNA interference

Introduction

Angiogenesis takes place during the course of development of the organisms, which is closely related to repair after trauma, ischemic and hypoxic injury, inflammation as well as the occurrence, progression and outcome of various diseases [1, 2]. Given the complex mechanism of angiogenesis, it is of high significance to identify the key targets. Endothelial cells are the main cells involved in the angiogenesis for which tube formation is an important step as a result of the interaction between the vascular endothelial cells (VECs) and other cells. Neural stem cells (NSCs) are pluripotent stem cells with the potential of differentiation into nerve cells and glial cells [3]. It is generally believed that NSCs are not a specific type of cells but a group of cells sharing similar properties. The discovery of NSCs has changed the previous belief that the neurons cannot regenerate, and a new therapy utilizing NSCs can be applied to treat nervous system diseases. Study shows that NSCs have the effect of brain tissue repair by inducing angiogenesis, though the mechanism is still unclear.

Epidermal growth factor-like domain 7 (EGFL7) is a newly discovered vascular endothelial growth factor (VEGF) that plays the key regulatory role in tube formation in embryonic vascular development. EGFL7 is usually highly expressed in liver cancer and malignant glioma [4, 5]. Human EGFL7 gene is located at the end of long arm of chromosome 9, encoding the protein of about 30 KD which contains a secretory signal peptide sequence. Comprising an amino-terminal EMI domain, EGFL7 can regulate cell adhesion. EGF-like domain related to protein recognition and the carboxyl terminal rich in lysine and valine are highly conservative. We built the co-culture system of primary human neural stem cells and human umbilical vein endothelial cells (NSCs-HUVECs) to simulate the interaction between NSCs and VECs in vitro [6-11]. The expression of EGFL7 in the two cells was inhibited by RNA interference (RNAi) and using recombinant lentiviral vector. Then the adhesion capacity and tube formation capacity of the VECs were detected so as to understand the role of EGFL7 in angiogenesis.

Table 1. Primers for quantitative PCR

Primers	Sequences (5'-3')
EGFL7	F: TGTGGCTTCTGGTGTTGGC
EGFL7	R: CGTCGCAGGTGGTGAGGA
Actin	F: TGACGTGGACATCCGCAAAG
Actin	R: CTGGAAGGTGGACAGCGAGG

Materials and method

Cell line and cule culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Cell Bank of Shanghai Institutes for Biological Sciences and preserved in DMEM (Gibco) containing 1% penicillin/streptomycin (Gibco) and 10% FBS (Gibco). NSCs were isolated from human embryo specimen with embryonic age of 8-10 weeks donated by Xinqiao Hospital. The NSCs were preserved in DMEM/F12 (Gibco) containing 1% penicillin/streptomycin, 10% FBS, EGF (10 mg/L), bFGF (10 mg/L) and N2 additive. The cells were cultured at 37°C in a 5% humidified CO_2 incubator.

Identification of human NSCs

Immunohistochemistry staining was performed for the neurosphere-forming NSCs. The cells were grown on the glass slide and fixed in 4% paraformaldehyde, transparentized with PBS containing 0.5% Triton X-100 and sealed in 6% goat serum. The cells were cultured with Nestin primary antibody (1:20) at 4°C overnight and then with RBITC-labeled secondary antibody (1:200) at 37°C for 1 h. After dilution with DAPI, the cells were incubated in the dark for 5 min. The slide was sealed with anti-fluorescent mounting medium and observed under the confocal laser scanning microscope.

Detection of EGFL7 expression in cells

The HUVECs and NSCs were grown on the glass slides and fixed in 4% paraformaldehyde. The cells were transparentized with PBS containing 0.5% Triton X-100, treated with 3% H_2O_2 to eliminate the activity of endogenous peroxidase, and sealed in 6% goat serum. The cells were incubated with EGFL7 primary antibody (1:20) at 4°C overnight and then with biotin-labeled secondary antibody (1:200) at 37°C for 30 min. Alkaline phosphatase-labeled streptavidin working solution was added to treat the cells at 37°C for 30 min. DAB substrate was added for color development in the dark for 10 min. After counterstained with hematoxylin for 1 min and washed with water, the cells were dehydrated through alcohol gradient and transparentized with xylene. The glass slides were sealed with neutral balsam.

Construction of pLV3/shRNA/EGFL7 vector

shRNA primer was synthesized according to the sequence of EGFL7 gene: 5'-GATCCGTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAACTTTTTTG-3', 5'-AATTCAAAAAAGTTCTCCGAACGTGTCACGTTCTCTTGAAACGTGACACGTTCGGAGAACG-3'. After annealing and linearization, the primers were ligated to shuttle plasmid pLV3. The positive clones were picked and sequenced and named as pLV3/shRNA/EGFL7. pLV3/shRNA/EGFL7 vector and packaging plasmids pRsv-REV, pMDlg-pRRE and pMD2G (Tronolab) were extracted using NucleoBond Xtra Midi Plus (Macherey-Nagel). The 293T cells were inoculated to 15 cm culture dish and cultured overnight until reaching 80-90% confluence. The shuttle plasmid and the packaging plasmids were mixed at a certain proportion and diluted in 1.5 ml serum-free DMEM. Then 300 μl RNAi-Mate (Western Biotechnology) was diluted separately in 1.5 ml serum-free DMEM and stood at room temperature for 5 min. Then the two were combined and stood for 20-25 min. The culture medium for 293T cells was replaced by serum-free DMEM and added with the mixed plasmids dropwise. Then the cells were further cultured in an incubator for 4-6 h and the culture medium was replaced by complete medium. After further culture for 72 h, the supernatant was collected.

Purification of Lenti-shRNA/EGFL7 vector

The lentiviral vector obtained after transfection of the 293T cells was named Lenti-shRNA/EGFL7. The supernatant from the culture of the transfected cells was centrifuged at 4°C at 4000 rpm for 4 min and passed through a 0.45 μm filter. Into every 100 ml of the filtered supernatant 50 ml solution containing 2.5 M NaCl and 20% PEG8000 was added, followed by ice bath for 1 h to precipitate the viruses. Centrifugation was performed at 4°C at 12000 rpm for 20 min with supernatant discarded, and the precipitate was resuspended in 10 ml of 1.10 g/ml CsCl solution. After centrifugation at 2000 rpm for 2 h at room temperature, 1.30-1.40 g/ml viruses were collected into a dialysis bag

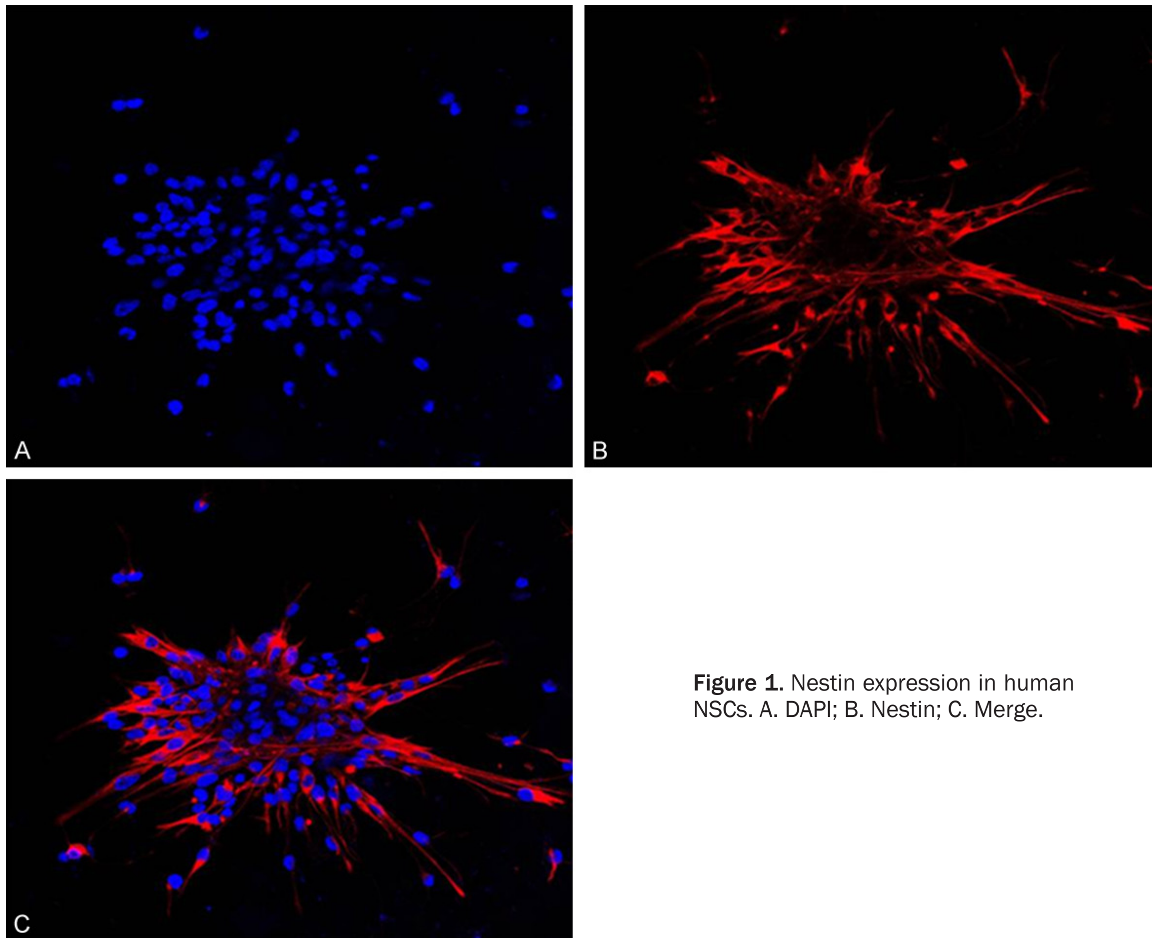

Figure 1. Nestin expression in human NSCs. A. DAPI; B. Nestin; C. Merge.

containing 10 mM Tris-HCl and 2 mM $MgCl_2$ (pH 8.0). Dialysis was performed at 4°C overnight, and the cells were collected, subpackaged and preserved at -80°C.

Titration of Lenti-shRNA/EGFL7 vector

The 293T cells were inoculated to a 96-well plate at 1×10^5 cells/well and placed in the incubator for 24 h. The purified Lenti-shRNA/EGFL7 vector was diluted by ten folds in DMEM, and 100 μl diluted virus solution was added into each well (10^{-2}-10^{-6}). The blank control group was set up and the cells were cultured in an incubator for 24-72 h. The positive cells were counted under the inverted fluorescence microscope. The virus titer was calculated according to the dilution fold (Transducing units per ml, TU/ml).

Transfection of NSCs-HUVECs by Lenti-shRNA/EGFL7 vector

First the 0.4 μm transwell membrane was coated with the culture medium for NSCs. Then HUVECs were inoculated to the 24-well plate at 1×10^4 cells/well. After the cells became adherent, the NSCs were inoculated to the transwell at 1×10^4 cells/well. The co-culture was carried out for 24 h. After that, the recombinant viruses were diluted with DMEM containing 8 μg/ml Polybrene (Sigma). The cells were infected at multiple of infection (MOI) of 15 and the culture continued for 72 h. The infection efficiency was observed under the inverted fluorescence microscope.

EGFL mRNA expression in HUVECs after infection

The HUVECs infected with Lenti-shRNA/EGFL7 vector were digested, centrifuged and collected. Total RNA was extracted using Trizol reagent (Qiagen). The primers used for reverse transcription and SYBR green I PCR (Eppendorf) are shown in **Table 1**. PCR conditions: 94°C for 4 min; 94°C for 20 sec, 60°C for 30 sec, 72°C for 30 sec, 35 cycles, with 3 replicates for each sample.

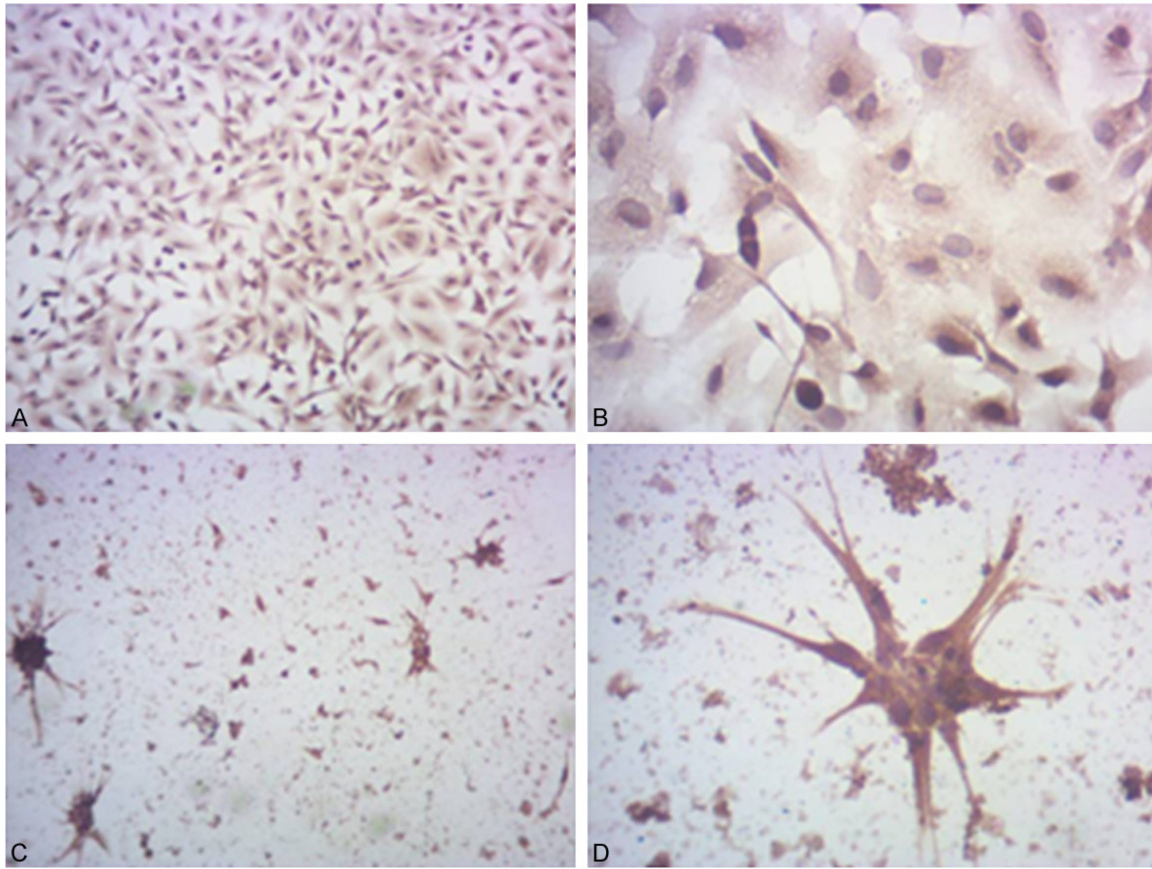

Figure 2. GFL7 expression in HUVECs and NSCs. A. HUVECs (100×); B. HUVECs (400×); C. NSCs (100×); D. NSCs (400×).

EGFL7 expression in HUVECs after infection

After co-culture of NSCs-HUVEC infected with Lenti-shRNA/EGFL7 vector, HUVECs were digested, centrifuged and collected, and washed with PBS once. Then RIPA lysis buffer was added, and the solution was mixed by vortex. The cells were resuspended and treated in an ice bath for 5 min. Centrifugation was performed at 4°C at 12000 g for 5 min, and the supernatant was collected and subjected to SDS-PAGE and Western Blot. The gels were scanned, and the gray values of the target bands were analyzed using Labworks 4.6 software (UVP).

Tube formation test of the transfected HUVECs

Matrigel (BD) was liquefied at 4°C for 12 h, and 200 μl Matrigel was added into each well of the 24-well plate to solidify for 30 min. The HUVECs after co-culture of the transfected NSCs-HUVECs were digested and inoculated to the plate at $4×10^4$ cells/well with 3 replicates. After further culture for 12 h, the tube formation was observed under the inverted microscope.

Detection of adhesion capacity of the transfected HUVECs

The HUVECs after co-culture of the transfected NSCs-HUVECs were digested and its concentration was adjusted to $1×10^5$ cells/ml. The cells were inoculated to the 24-well plate precoated with poly-L-lysine at 500 μl each well. After culture in the incubator for 30 min, the cells were washed with PBS, fixed in 4% paraformaldehyde at room temperature and air dried. Into each well 0.6 ml 0.1% crystal violet was added to stain the cells at room temperature for 20 min. Then the staining fluid was gently removed. Each well was washed with distilled water for 3 times and air dried, and 0.6 ml 33% acetic acid was added into each well for decolorization with proper mixing. Then 150 μl of solution was collected from each well and added into the 96-well plate. The OD value was measured at 570 nm and the blank control group was set up.

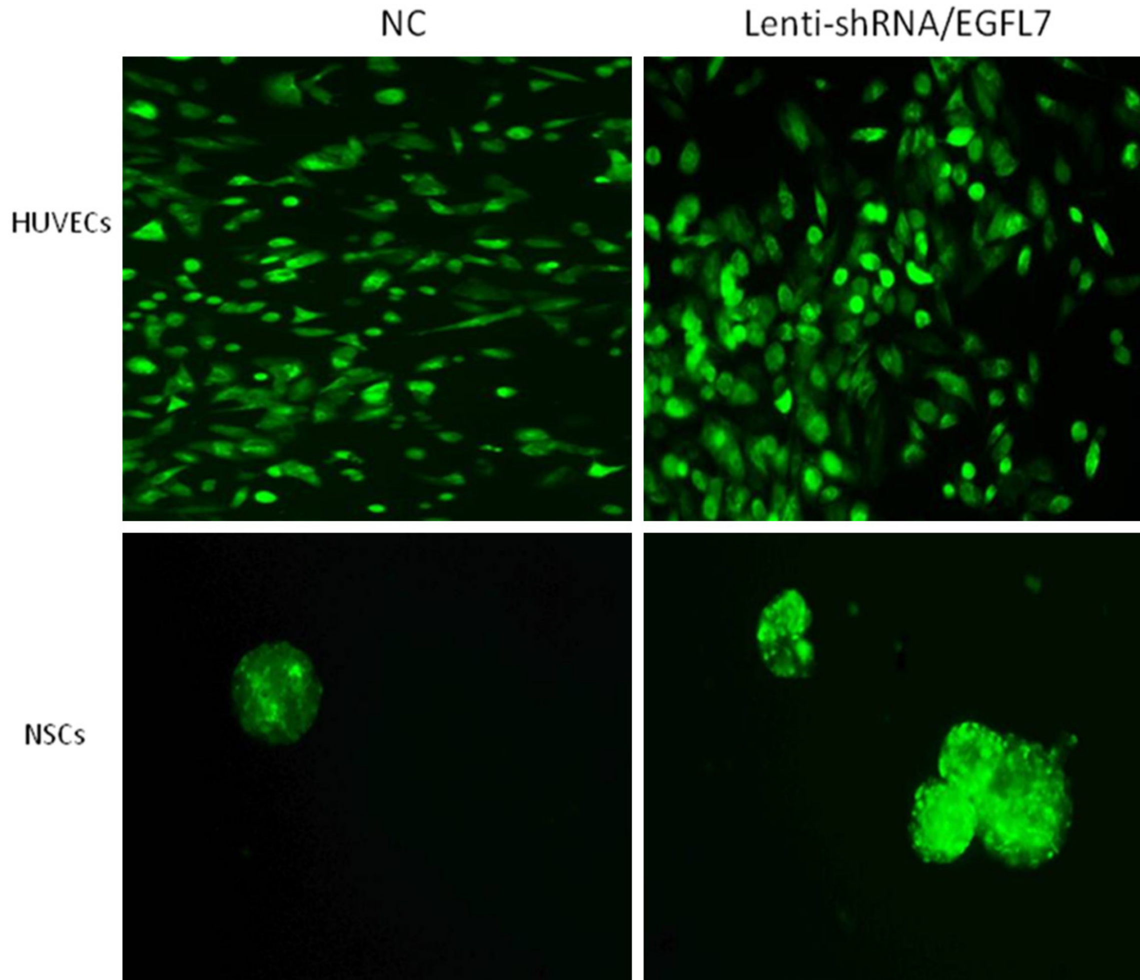

Figure 3. Transfection of HUVECs-NSCs by Lenti-shRNA/EGFL7 vector.

Statistical analysis

Three replicates were set for each experiment. Statistical analysis was performed using SPSS 10.0 software and the means of two samples were compared with t-test.

Results

Identification of the cells and factor expressions

Immunohistochemistry staining was combined with confocal laser scanning microscopy. Nestin was expressed in the cytoplasm of the primary cells isolated from the human embryo specimen (**Figure 1**), and the cells were identified as human NSCs. Immunohistochemistry was performed to detect the expression of factors in HUVECs and NSCs. The results showed that EGFL7 was stably expressed in both HUVECs and NSCs (**Figure 2**).

Transfection of HUVECs-NSCs by Lenti-shRNA/EGFL7 vector

The titer of the purified Lenti-shRNA/EGFL7 vector was $1×10^8$ TU/ml. The recombinant lentivirus not containing the exogenous gene was prepared as the negative control by the Western Biotechnology, with viral titer of $5×10^8$ TU/ml. The two viruses were used to infect the HUVECs-NSCs after co-culture at MOI = 15, respectively. After 96 h, the infection efficiency was both 100% for either virus in the co-cultured cells. As shown in **Figure 3**.

Silencing of EGFL7 gene by RNAi

The dissolution curves indicated good specificity amplification of both EGFL7 gene and

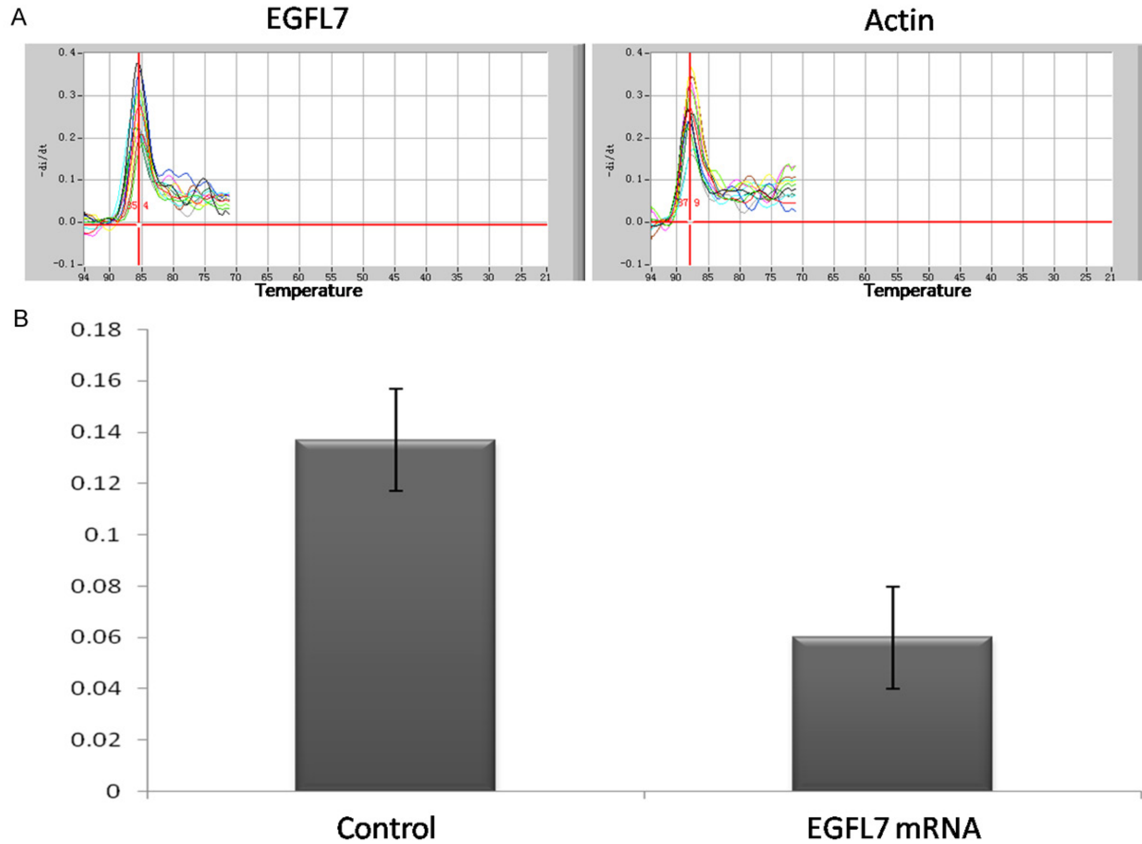

Figure 4. A: Dissolution curves of EGFL7 and actin in quantitative fluorescence PCR; B: Relative quantitative results of EGFL7 mRNA levels after RNAi.

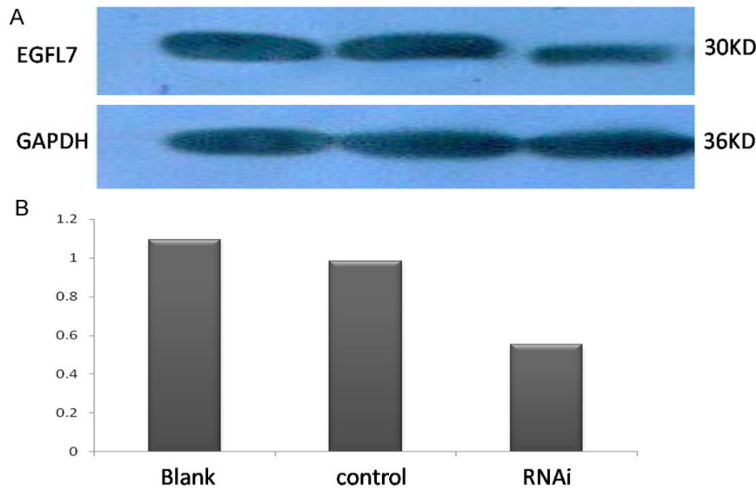

Figure 5. Detection of EGFL7 expression after RNAi by Western blotting. A. Western blotting; B. Relative quantitative results of EGFL7 mRNA levels after RNAi.

actin as the internal reference (**Figure 4A**). The standard curves showed that the amplification efficiency of the two PCR systems was all higher than 95%. After transfection of HUVECs by Lenti-shRNA/EGFL7, the mRNA expression level of EGFL7 gene was decreased significantly as compared with the negative control according to relative quantification (P<0.05). Thus the RNAi sequence could effectively silence the target gene (**Figure 4B**).

Decreased EGFL7 expression after RNAi

Western Blot and gray value analysis indicated that the EGFL7 expression in the transfected HUVECs after co-culture in the blank group and the control group did not decrease significantly (P>0.05); in contrast, the EGFL7 expression in the transfected HUVECs after co-culture in the experimental group decreased greatly (P<0.05) (**Figure 5**).

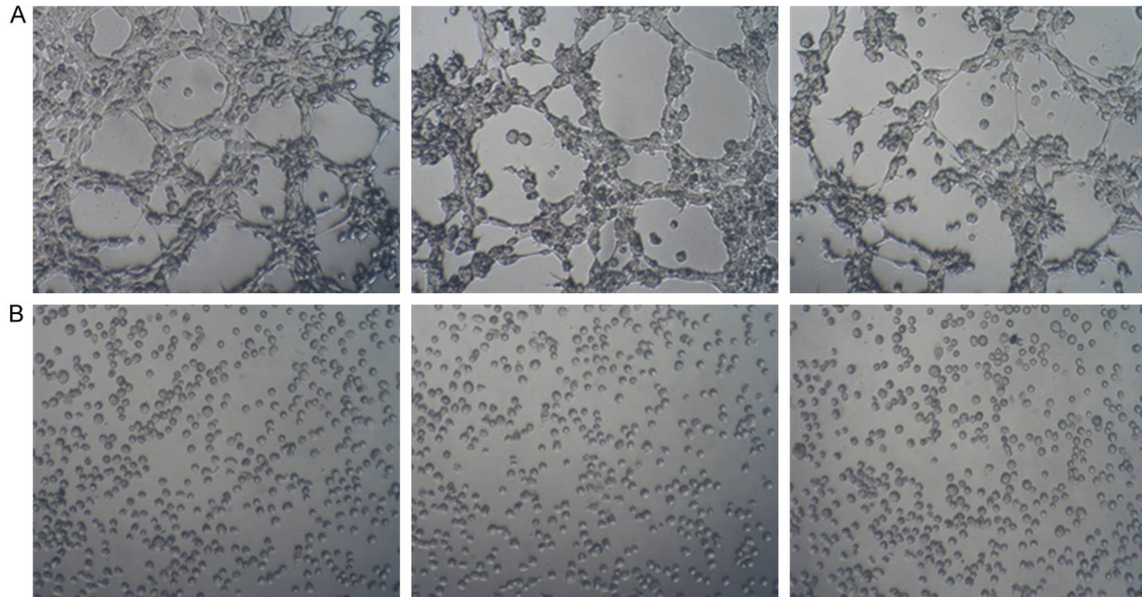

Figure 6. A. Tube formation capacity of HUVECs in vitro after RNAi; B. Adhesion capacity of HUVECs in vitro after RNAi.

Table 2. Eluent OD570 adhesion test results after the detection of crystal violet staining

Groups	OD1	OD2	OD3	Mean OD
Blank	0.6739	0.6561	0.6849	0.6716
Control	0.6052	0.6222	0.6254	0.6176
RNAi	0.3973	0.3849	0.4008	0.3943

Decreased tube formation capacity of HUVECs after RNAi

Under normal culture conditions, the HUVECs would elongate and form the cord-like and the reticular structures with lumens of varying size and shape (**Figure 6A**). After transfection of the HUVECs-NSCs, the number of lumens in the HUVECs receiving RNAi (experimental group) (10.2±4.6) was obviously lower than that in the control group transfected with empty vector (24.4±3. 0) (P<0.05) and in the normal cells (blank group) (22.6±2.6) (P<0.05). There was no obvious difference between the control group and the blank group (P>0.05).

Decreased adhesion capacity of HUVECs after RNAi

The number of HUVECs receiving RNAi (experiment) adhering to the cell plate precoated with poly-lysine was much lower than that in the cells transfected with empty vector (control group) and in the normal cells (blank group) (see **Figure 6B**). After decolorization with acetic acid, the OD value was measured at 570 nm. As shown in **Table 2**, the OD value of the experimental group was significantly different from that of the control group and the blank group (P<0.05); there was no obvious difference between the control group and the blank group (P>0.05). This indicated that the adhesion capacity of HUVECs decreased remarkably after RNAi.

Discussion

EGFL7 is a recently discovered endothelial cell-derived secretory factor with high expression in embryo and tissues rich in blood vessels but very low expression in most mature tissues. EGFL7 can greatly enhance the migration capacity of endothelial cells and fibroblasts [12]. In the present study, we found high expression of EGFL7 in primary human NSCs by immunohistochemistry and RT-PCR. This suggested the non-specificity of EGFL7 expression. We established the co-culture system of NSCs and HUVECs using transwell technique and simulated the interactions between NSCs and VECs in vitro. The recombinant lentiviral vector targeting EGFL7 gene and containing green fluorescent protein was constructed using RNAi technique. This vector achieved effective sil-

encing of EGFL7 gene in NSCs and HUVECs. After silencing of EGFL7 gene, the adhesion capacity of HUVECs was greatly inhibited. Tube formation assay suggested that no tubes were formed by VECs after the silencing of EGFL7 gene. All these findings confirmed that EGFL7 played the key regulatory role in angiogenesis induced by NSCs by regulating the adhesion of VECs.

According to other experiment, after the silencing of EGFL7 gene in *Brachydanio rerio*, no tubes were formed during embryonic development, indicating the significance of EGFL7 in tube formation [13]. Therefore, EGFL7 may be the potential target in anti-angiogenesis therapy. Most of the existing studies on EGFL7 are concerned with its role in embryonic development and repair of vascular injury [14-16]. We found in the present study that the angiogenesis of HUVECs induced by primary human NSCs was greatly inhibited after the silencing of EGFL7 gene. This confirmed the regulatory effect of EGFL7 gene in angiogenesis. The adhesion and integration of VECs to the extracellular matrix are crucial to angiogenesis, and we found that the silencing of EGFL7 caused the decreased adhesion capacity of HUVECs. Other reports said that EGFL7 gene promoted the adhesion of HUVECs. However, it can be certain that EGFL7 can regulate the adhesion of VECs [17, 18].

In this study, after silencing of EGFL7 gene in NSCs and HUVECs by RNAi, the angiogenesis of HUVECs induced by NSCs was greatly inhibited. The action mechanism may be that EGFL7 regulates the adhesion and migration of VECs and affects the tube formation. EGFL7 gene may be the candidate target gene in the anti-angiogenesis therapy.

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Disclosure of conflict of interest

None.

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