Supporting information of

From single to dual-gene delivery nanosystem: coordinated expression matters for boosting neovascularization in vivo

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Materials

Multifunctional peptidyl carrier REDV-TAT-NLS-H_{12} (complete amino acid sequence: REDVYGRKKRRQRRRPKKKRKVHHHHHHHHHHHH, noted as RH-34) was synthesized from GL Biochem. (Shanghai) Ltd. ZNF 580 plasmid and $VEGF_{165}$ plasmid were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen Corporation (Carlsbad, CA). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-

tetrazolium bromide (MTT) and Cell Counting Kit-8 (CCK-8) were purchased from Ding Guo Chang Sheng Biotech. Co., Ltd. (Beijing, China). Rabbit anti-human ZNF_{580} polyclonal antibody, rabbit anti-human $VEGF_{165}$ polyclonal antibodygoat, anti-rabbit IgG were purchased from Abcam Ltd. (Shanghai, China). Human Human umbilical vein endothelial cells (HUVECs) were acquired from the Cell Bank of Typical Culture Collection of Chinese Academy of Sciences (Shanghai, China). Male mice (6 weeks old, 20 - 25 g) were provided by the Department of Physiology and Pathophysiology, Logistics University of Chinese People's Armed Police Force. Before experiments, they were hosted in an SPF fully enclosed animal feeding room with standard animal experimental facilities.

Measurement with size and Zeta potential

The size and zeta potential of single and dual-gene complexes in PBS with different weight ratios of RH-34/pDNA were analyzed through Zetasizer Nano ZS (Malvern Instrument, Inc., Worcestershire, UK).

Agarose gel electrophoresis

In short, 10 μL of gene delivery with different weight ratios were mixed with 2 μL of loading buffer (6×). And then, the above obtained samples were loaded into the 0.8 % agarose gel containing 6 μL of GoldView with 1 × TAE buffer at 120 V for 25 min. Finally, UV illuminator was performed to visualize the plasmids and evaluate the plasmid loading ability.

Cell culture and in vitro transfection

HUVECs were cultivated in 6-well plates with complete medium to until 80% cell fusion approximately. After starving 12 h, various single and dual-gene delivery systems were respectively added into each well, 5μg plasmid per well. The remaining well added to the same volume of 0.01 M PBS (pH=7.4) was used as a blank control. Then, the medium was replaced with complete medium after 4 h incubation and the cells continued to culture for 24 h in incubator.

In vitro cytotoxicity

MTT assay was performed to evaluate the cytotoxicity of RH-34 gene vector and gene delivery system based on pZNF580 (w/w ratio = 5). In brief, HUVECs (1×10^4 cells/well) were planted in and cultured in DMEM containing 10% FBS for 24 h to achieve 80 % of confluence. And then the medium was replaced into serum-free one and cultured for overnight. Different concentrations of RH-34 and single-pZNF580 delivery system solutions were added and incubated for another 48 h. MTT reagent (20 μL, 5 mg/mL) was added into each well and culture for another 4 h to form formazan crystals. The medium was removed, followed by adding 150 μL of DMSO for dissolving crystals. The optical density was measured at 490 nm using a microplate reader (BIO-RAD, iMark™, USA). The relative cell viability was calculated as:

$$\text{relative cell viability}(\%)=\frac{OD_{490}(\text{sample})}{OD_{490}(\text{control})}\times 100\%$$

Real-time quantitative PCR

Real-time quantitative PCR (qPCR) was performed to estimate mRNA expression of HUVECs, which verified the transfection efficiency at the level of mRNA. At 24 h after transfection as described above procedure (equivalent 5μg of total plasmids each well), HUVECs were washed three times with 0.01 M PBS (pH=7.4) and lysed by 500 μL TRIzol reagent per well to extract the total RNA. Then, the RNA was reverse-transcribed into cDNA, which was further used to complete realtime fluorescence quantitative PCR assay on 7500 Real-Time PCR System. The expression of house-keeping gene GAPDH was used as an internal control. The PCR forward and reverse primer sequences of GAPDH, ZNf580 and VEGf165 were as follows: GAPDH forward 5'-AGGTGAAGGTCGGAGTCAAC-3' , GAPDH reverse 5'-CGCTCCTGGAAGATGGTGAT-3' and ZNF_{580} forward 5'-AAAAAGCTTGTGGAGGCGCACGTGCTG-3', ZNF_{580} reverse 5'-AAAAAGATCTTGCCCGGAGTGCGCCCGTG-3' and $VEGF_{165}$ forward 5'-AGGCCAGCACATAGGAGAGA-3', $VEGF_{165}$ reverse 5'-

CAAGGCCCACAGGGATTTT-3' respectively. The results were analyzed using StepOne software v2.1.

Western blot analysis

Western blot assay was used to detect the ZNF580 and VEGF165 protein expression level of different pDNA gene in HUVECs. In brief, adding the protein lysate RIPA and PSMF (v/v = 100/1) to HUVECs transfected (equivalent 5μg of total plasmids each well) after 24 hours for 30 min on ice. The total protein was obtained in supernatant after further ultrasonic rupture and centrifugation at 1,3000 rpm at 4 °C for 6 min. Then, quantified protein and loading buffer (5×) were configured as a mixture for 12% SDS-PAGE electrophoresis. After a 60 min transfer, the proteins were transferred onto polyvinylidene difuoride (PVDF) membranes, which were further sealed with 5% milk for 2 h and cultured respectively in anti-ZNF580 and anti-VEGF165 antibody in TBST overnight at 4 °C. In the meanwhile, the β-actin protein was used as a control. After that, the membranes incubated with anti-rabbit secondary antibody for 2 h. Subsequently, before observed by a gel image analysis system, the membranes were washed three times with TBST and developed via ECL Western Blotting Substrate Kit. Furthermore, PVDF was washed in a shaker for 30 min, incubated with horseradish peroxidase conjugated to goat antirabbit IgG to assess the protein loading level, and coated with enhanced chemiluminescent reagents to exposure. The bands were visualized using ImageJ 2.

Cell migration assay

The migration capability of the transfected HUVECs by different gene complexes was quantified by the wound healing assay and transwell migration assay. Monolayer HUVECs were seed in a 6-well plate and transfected with various gene delivery systems as above describe procedure (equivalent 5μg of total plasmids each well). Cultivating for another 24 h, the cells were mechanically scratched by 200 μL micropipettor tip. Then, cellular debris was washed away by D-Hanks. The wound healing was observed and recorded by an inverted microscope at 0, 6 and 12 h.

The migration ability was further evaluated by calculating relative recovered area (%) using Image J software by the following equation.

$$\text{Migration area (\%)} = (\text{wounded area} - \text{nonrecovered area})/(\text{wounded area}) \times 100$$

The transwell migration assay was also performed to investigate cell migration ability by using the transwell chambers with pore (8 μm aperture). After transfected by equivalent 5μg of total plasmids each well for 24 h culture, HUVECs digested with trypsincells were washed twice using D-Hanks and seeded in chambers with serum-free medium (2.0×10^4 cells per well). In the meanwhile, the complete medium was added in the lower transwell chambers and the transwell system was incubated for another 6 h. After that, the chambers were taken out and washed three times with PBS (pH=7.4) buffer and fixed 10 min with 4% formaldehyde fixative (pH=7.4). The cells on the upper surface that didn't pass through the pore of chambers were removed by sterile cotton swabs. Furthermore, Crystal Violet Staining Solution was used to dye the cells on the lower surface for 5 min. Then, the cells passed through pores were observed by microscope and counted by Image-Pro Plus 6.0 software.

Cell proliferation assay

The proliferation capability of HUVECs was detected by CCK-8 assay. The cells were seeded in 96 well plate at 7000 cells per well following the instructions while the different gene delivery systems added at a concentration of 2.5 μg/mL (equivalent 5μg of total plasmids each well). Set four duplicate wells per sample to improve the accuracy. The cells were added 10 μL CCK-8 reagent after 12, 24, 48 h and were further cultured for 2 h. After that, the absorbances at 490 nm measured by a microplate reader showed the HUVECs proliferation.

Tube formation assay in vitro

In order to evaluate the angiogenic function of HUVECs transfected by different gene complexes, tube formation assay in vitro was performed. Firstly, matrigel was dissolved

in advance at 4 °C overnight, in the meanwhile, the 96-well plate and the pipette tips were pre-cooled at - 20 °C. Then, the required wells in 96-well plate were coated with 60 μL matrigel and incubated at 37 °C for 30 min. Groups were designed as in vitro transfection assay. The transfected cells (equivalent 5μg of total plasmids each well) in 6-well plate were digested and seeded on the solidified matrigel (4×10^4 cells per well) for another 6 h incubation. Then, the images of tubes formation were observed and photographed by a microscope and the number of formed tubes were analyzed using Image J 2.1.

Tube formation assay in vivo

For further examining the capacity of different REDV-TAT-NLS-H_{12}/pDNA complexes on tube formation, the angiogenesis assay in vivo was performed. SD mice (male, 5 weeks old, 20−25 g) were used as an experimental model. Firstly, HUVECs were transfected as described before procedure (equivalent 5μg of total plasmids each well) and the groups were designed as vitro transfection assay. Then, the cells were trypsinized and mixed with 400μL matrigel to reach a final cell concentration of 1×10^6 cells mL^{-1}. Mice were injected anesthetic before injected the mixture into the abdominal cavity using a 26-gauge needle. After four days, matrigel implants were taken from the anesthetized mice and fixed with neutral formaldehyde. Sections were subsequently blocked with 5% BSA (v/w) in Tris-buffered saline (TBS) on ice. The number of microvessel structure was confirmed by immunofluorescence staining of the HUVECs membrane marker CD31. All animal experiments conformed to the guidelines of the Council for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Public Health, China and the Guidelines for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

Table S1. The contents of pZNF580 and pVEGF165 plasmids per well in biological experiments

Gene delivery systems	pZNF580	pVEGF165	Total

		per well	per well	plasmids per well
Single-gene delivery systems	Single-pZNF580 delivery system	5 μg	0 μg	5 μg
	Single-pVEGF165 delivery system	0 μg	5 μg	5 μg
Dual-gene delivery systems	Dual-p1:2 delivery system	1.67 μg	3.33 μg	5 μg
	Dual-p2:1 delivery system	3.33 μg	1.67 μg	5 μg

Note: biological experiments include PCR, western blot, CCK-8, transwell, wound healing, in vitro and in vivo angiogenesis assays.

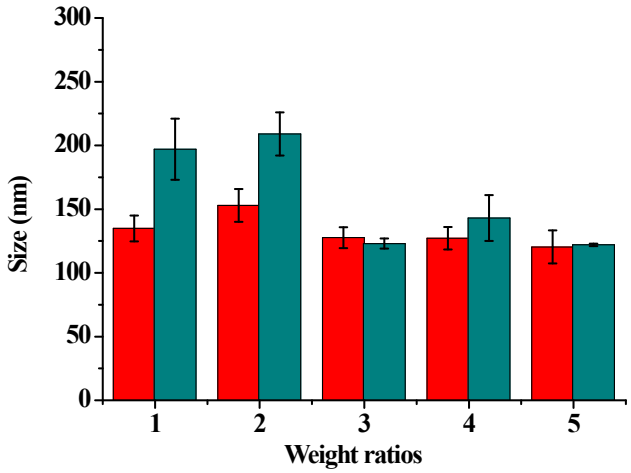

Figure S1. The size of single-gene delivery systems in PBS (pH = 7.4) with different weight ratios of RH-34/pDNA. The red and dark cyan columns represented single-pVEGF165 delivery system and single-pZNF580 delivery system, respectively. (n = 3, mean ± SD).

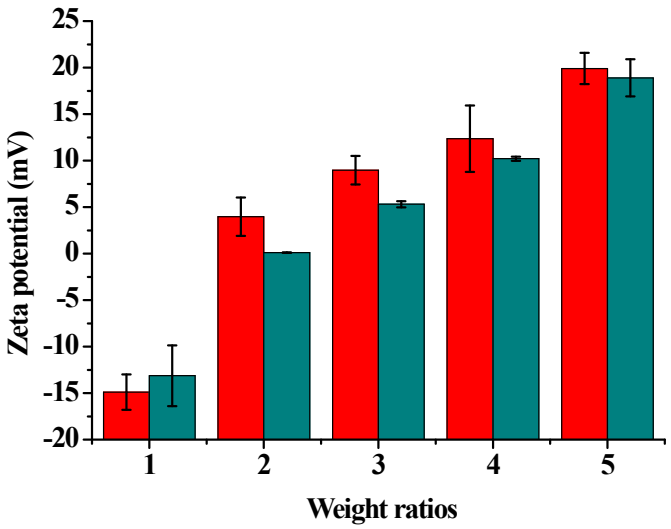

Figure S2. The zeta potential of single-gene delivery systems in PBS (pH = 7.4) with different weight ratios of RH-34/pDNA. The red and dark cyan columns represented single-pVEGF165 delivery system and single-pZNF580 delivery system, respectively. (n = 3, mean ± SD).

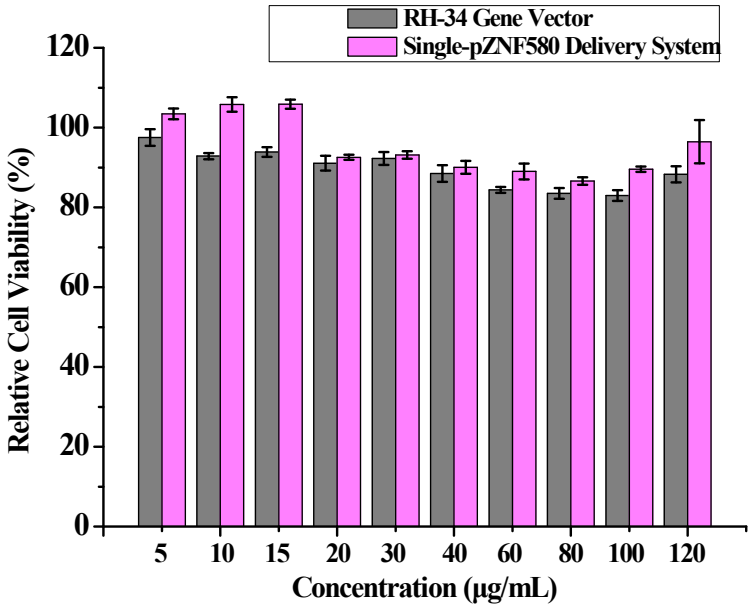

Figure S3. Relative cell viability of HUVECs after treatment with different concentrations of RH-34 gene vector and single-pZNF580 delivery system for 48 h by MTT assay. (n = 3, mean ± SD).