Homocysteine inhibits the viability and migration ability of human umbilical vein endothelial cells by downregulating the expression of vascular endothelial growth factor

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Abstract. The current study aimed to explore the effect of homocysteine (Hcy) on the viability and migration ability of human umbilical vein endothelial cells (HUVECs), as well as to examine the underlying mechanism. The association between the expression level of Hcy and lower extremity deep vein thrombosis (DVT) was detected in clinical samples collected from patients. In addition, the effect of Hcy on the viability and migration ability of HUVECs was detected by cell counting kit-8 and Transwell assays, respectively, while vascular endothelial growth factor (VEGF) expression was measured in order to verify the effect of Hcy on VEGF. The results indicated that the serum Hcy levels in DVT patients were significantly increased. *In vitro* experiments also confirmed that Hcy was able to significantly inhibit the viability and migration ability of HUVECs, and downregulate the expression of VEGF in these cells. Furthermore, the inhibitory effect of Hcy on HUVEC viability and migration ability was achieved by downregulating the expression of VEGF using small interfering RNA transfection. In conclusion, Hcy inhibited the viability and migration ability of HUVECs by downregulating the expression of VEGF. This may underlie the high incidence of DVT in patients with hyperhomocysteinemia.

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Introduction

Lower extremity deep vein thrombosis (DVT) is a common venous disease in clinical practice. The pathogenesis of this disease is considered to involve three factors that were first suggested by the German pathologist Rudolf Virchow in 1865. These three factors include poor blood circulation, vascular intima injury and alterations in blood composition caused by various reasons, such as hypercoagulability (1,2).

The role of homocysteine (Hcy) in the pathogenesis of DVT has been reported in a large number of studies (3-6). Hcy, an amino acid and an important intermediate product in the metabolism of methionine and cysteine, is associated with vascular injury (7,8). Hcy is unstable and prone to oxidization into Hcy or Hcy-Cys disulfide (9,10). Furthermore, a small amount of Hcy is present in the plasma in a reduced form. Hcy has been reported to be a possible risk factor of vascular endothelial injury and hypercoagulability (11,12). A previous epidemiological study revealed that ~25% of patients with venous thromboembolism exhibited elevated levels of Hcy to different extents, while one third of patients with venous thromboembolism developed hyperhomocysteinemia (8,9). Additionally, Hcy may be involved in thrombosis by damaging the vascular endothelium, activating platelets and the coagulation system, and inhibiting protein C (11-13). However, the specific mechanism underlying the damage of endothelial cells caused by Hcy remains unclear.

In the present study, the serum Hcy levels of 96 patients were analyzed, and hyperhomocysteinemia was observed to be closely correlated with DVT. Furthermore, human umbilical vein endothelial cells (HUVECs) were stimulated with different concentrations of Hcy, in order to investigate the effects of Hcy on the viability and migration ability of HUVECs. It was further confirmed that Hcy functioned by downregulating the expression of vascular endothelial growth factor (VEGF).

Materials and methods

Clinical data. The study group comprised of 96 patients with DVT, who were treated at Qianfoshan Hospital of Shandong University (Jinan, China) between December 2014 and March

2016. Among these, 46 patients were male and 50 were female. The age of these patients ranged between 33 and 79 years, with a mean age of 52.3±6.5 years. Definitive DVT diagnosis of these patients was established by color Doppler ultrasound (14). In addition, 80 subjects were recruited into the control group, including 38 male and 42 female subjects, with an age ranging between 32 and 75 years and a mean age of 53.5±5.6 years. These control subjects were healthy based on physical examinations. Patients with atrial fibrillation, diabetes, liver and kidney dysfunction, cerebral hemorrhage, cerebral infarction, thyroid dysfunction and malignant tumors were excluded from the two groups. Individuals who took a variety of vitamins and had recently used anticoagulation and/or hemostatic drugs were also excluded. Differences in the age, gender, body weight and other characteristics of the two groups were not statistically significant.

Elbow vein blood samples were collected in vacuum tubes from all subjects at a fasting state in the morning after hospitalization or on the day of outpatient service. Detection of Hcy was completed on the same day following serum separation. Serum Hcy levels were measured by high-sensitivity immunoturbidimetry as previously described (3).

Written informed consent was obtained from patients prior to participation, and the Ethics Committee of Qianfoshan Hospital of Shandong University (Jinan, China) provided approval for the present study.

Reagents. The M199 culture medium was purchased from GE Healthcare Life Sciences (HyClone; Logan, UT, USA). Fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), cell culture plates (6-well and 24-well), cell counting kit-8 (CCK-8) and the VEGF-A ELISA kit (eBioscience) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Transwell chambers (0.4 μm in diameter; 24-well type) were purchased from Corning, Inc. (Corning, NY, USA). The Total RNA extraction kit, RNA reverse transcription (RT) kit, and quantitative polymerase chain reaction (qPCR) reaction system, which included: 2.0 μl dNTP, 2.5 μl 10xPCR buffer, 0.15 μl Taq Hot Start, 2.0 μl genomic DNA and 0.5 μl 10 μM forward and reverse primers, were purchased from Tiangen Biotech Co., Ltd. (Beijing, China). The VEGF antibody (cat. no. ab69479; 1:500) was purchased from Abcam (Cambridge, UK). The anti-mouse secondary antibody (1:2,000) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA; cat. no. 7076P2). Analytical grade, pure Hcy was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), while recombinant human VEGF (with a working concentration of 5 ng/ml) was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). Crystal violet dye was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Primers used in qPCR were synthesized by BGI (Shenzhen, China).

Instruments and equipment. Determination of serum Hcy levels was conducted using an automatic biochemical analyzer (Olympus Corp., Tokyo, Japan), and the corresponding reagents and standards were provided by Abbott Pharmaceutical Co. Ltd. (Lake Bluff, IL, USA). Other instruments included a gel imaging device (GelDoc 2000; Bio-Rad Laboratories, Inc., Hercules, CA, USA), PCR machine (Mastercycler nexus; Eppendorf, Hamburg, Germany) and image-enabled inverted optical microscope (CKX31; Olympus Corp.). Experiments were conducted strictly in accordance with the protocols provided by the instrument manufacturer.

HUVEC collection and culture. HUVECs were obtained from the umbilical cord of healthy full-term fetuses delivered by cesarean section at Qianfoshan Hospital of Shandong University. Briefly, the umbilical veins were picked subsequent to washing with phosphate-buffered saline (PBS). Next, 0.25% trypsin was injected into the lumen of the veins and incubated for 8-10 min at 37˚C. The digestive juice in the veins was then collected, and M199 medium containing 20% FBS was added to rinse the lumen. Subsequently, the aforementioned fluids were collected, placed into a 50-ml centrifuge tube and centrifuged at 1,200 x g at 4˚C for 5 min. The supernatant was discarded, complete medium (M199 medium containing 20% FBS) was added and homogenized by shaking, and the cells were counted using a counting plate. Cells were then cultured in a 25-cm^2 culture flask at a density of 2-5x10^5 cells/ml in an incubator with 5% CO_2 at 37˚C. After 24 h of culture, the medium was replaced by fresh M199 medium containing 20% FBS, and 2.5 μl/ml recombinant VEGF was added. Cells in 3-10 generations were collected for use in subsequent experiments. HUVECs were evaluated using VIII factor related antigen staining. HUVECs were fixed using 4% paraformaldehyde (dissolved in PBS) at 25˚C for 5 min and stained by VIII antibody (1:200; cat. no. ab78852; Abcam), then incubated at 37˚C for 1 h. After washing with PBS, related Alexa Fluor® conjugated secondary goat-anti-mouse antibody (1:1,000; cat. no. A-11029; Invitrogen; Thermo Fisher Scientific, Inc.) was added and the expression of the VIII factor was observed using fluorescence microscope (magnification, x200).

Cell transfection. At 1 day prior to transfection, the HUVECs were seeded into 6-well plates at a density of 1.5-3x10^5 cells/well. When 30-50% confluence was reached, cells were transfected with VEGF-small interfering RNA (siRNA) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 20 min. VEGF-siRNA was designed and synthesized by Sigma-Aldrich (Merck KGaA, Darmstadt Germany), and the sequences were as follows: Antisense, 5'-AAAGUAGUGCUUCACCACUUC-3', and sense, 5'-UCAUCACGAAGUGGUGAAGAA-3'. The negative control was also provided by Sigma-Aldrich (Merck KGaA), with the following sequence: Forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3'.

RT-qPCR. Total RNA was extracted using chloroform from the collected HUVECs. Subsequent to determining the RNA concentration using a spectrophotometer (Bio-Rad Laboratories, Inc.), total RNA was reversely transcribed into cDNA using an RT kit, and then subjected to qPCR using STBR-Green (Tiangen Biotech Co., Ltd.). The primer sequences used were as follows: VEGF, 5'-CGCAAGAAATCCCGGTATAAGTCC-3' (upstream), and 5'-CGTTCGTTTAACTCAAGCTGCCTC-3' (downstream); GAPDH, 5'-AACGGATTTGGTCGTATTGGG-3' (upstream), and 5'-CCTGGAAGATGGTGATGGGAT-3' (downstream). GAPDH served as the internal control, while VEGF was the target molecule.

The reaction conditions of PCR amplification consisted of pre-denaturation at 95˚C for 5 min, denaturation at 95˚C for 30 sec, annealing at 58˚C for 30 sec, and extension at 72˚C for 30 sec, with a total of 30 cycles performed.

Western blot analysis. Total proteins were extracted using radioimmunopricipitation assay buffer (Beyotime Institute of Biotechnology) from the HUVECs, and the protein concentration was determined using the bicinchoninic acid method. Next, 40 μg total protein was isolated by sodium dodecyl sulfate-polyacrylamide gel (10%) electrophoresis, transferred onto a polyvinylidene difluoride film and incubated with 5% bovine serum albumin at room temperature for 1 h in order to block nonspecific binding. Subsequently, the VEGF primary antibody was added and incubated at 4˚C overnight. The film was then washed three times with Tris-buffered saline and Tween-20 for 10 min each time, followed by addition of the corresponding secondary antibody and incubation for 1 h at 37˚C. Following further washing for three times (10 min each), VEGF expression was detected by enhanced chemiluminescence (EMD Millipore, Billerica, MA, USA), and the gray value was analyzed using Quantity One 4.62 software (Bio-Rad Laboratories, Inc.).

Detection of cell viability by CCK-8 assay. The concentration of HUVECs was adjusted to $3.0x10^4$ cells/ml, and cells were cultured in 96-well plates at a density of 3,000 cells/well in an incubator with 5% CO_2 at 37˚C for 24 h. Subsequently, different concentrations of Hcy were added to untransfected and transfected cells, and then 10 μl CCK-8 reagent was added to each wells at various time points, including at 0, 24, 48 and 72 h. Subsequently, the samples were homogenized by shaking and placed in an incubator for 1 h at 37˚C. Finally, the optical density value was measured at a wavelength of 450 nm.

Detection of the invasion ability of cells by a Transwell assay. HUVECs were prepared into a single-cell suspension using trypsin (Beyotime Institute of Biotechnology) and the number of cells was counted. Following centrifugation at 1,200 x g at 4˚C for 5 min, the supernatant was discarded, and cells were resuspended the cells using M199 medium containing 1% FBS. Next, the cell concentration was adjusted to $1x10^6$/ml. In a sterile 24-well plate, 600 μl double antibody-free M199 medium (containing 10% FBS) was added to the wells, and the Transwell chambers were placed into the wells. Subsequently, 100 μ of the aforementioned cell suspension was placed into the prepared chambers, and the plate was placed in an incubator with 5% CO_2 at 37˚C for 12 h. The solution in the upper chambers was discarded, and the chambers were washed with PBS to remove culture medium and other impurities. The collected cells were fixed with methanol for 10 min and stained with crystal violet for 20 min. Cells in the chambers on the upper layer of the membrane were cleaned with a cotton swabs. A total of five visual fields were selected under a microscope, and the number of cells in each field was counted.

Detection of VEGF-A expression level by ELISA. HUVECs were collected, and the cell concentration was adjusted to $1x10^5$/ml, followed by culture in 24-well plates at a density of 500 μl/well in an incubator with 5% CO_2 at 37˚C. After the cells were treated with different concentrations of Hcy for 24 h, serum-free M199 medium was added and cells were incubated for a further 24 h. The supernatant was collected, centrifuged at 1,000 rpm for 10 min in order to remove the cell mass, and then centrifuged at 12,000 rpm for 10 min to remove the cell debris. The supernatant was collected and used to determine the content of VEGF-A by an ELISA kit (cat. no. JN-03012; R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer's protocol.

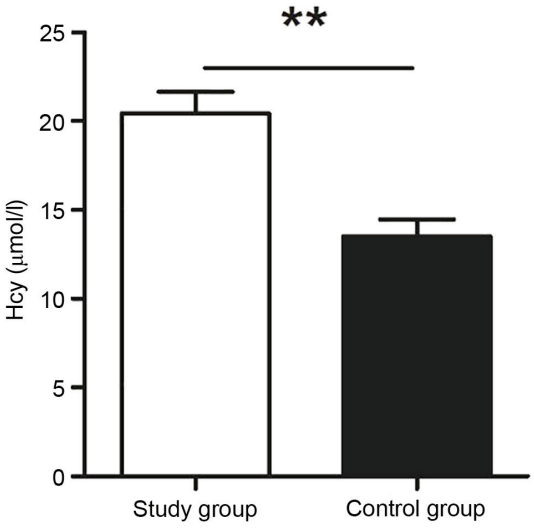

Figure 1. Comparison of serum Hcy levels between DVT patients and healthy controls. **P<0.01. Hcy, homocysteine; DVT, deep vein thrombosis.

Statistical analysis. Data were statistically analyzed using SPSS version 16.0 software (SPSS, Inc., Chicago, IL, USA. Measurement data are expressed as the mean ± standard deviation. Intergroup comparison was conducted using Student's t-test or χ^2 test. P<0.05 was considered to indicate a difference that was statistically significant.

Results

Serum Hcy levels are significantly increased in DVT patients. Serum Hcy levels in samples obtained from DVT patients and control subjects were compared using a Student's t-test. The results revealed that Hcy levels in the serum of DVT patients were significantly increased when compared with those in the controls (Fig. 1).

Hcy significantly inhibits the viability of HUVECs. Previous studies have revealed that Hcy may promote the formation of DVT by damaging the vascular endothelium (4,5). In order to confirm the effect of Hcy on endothelial cells, the effect of different concentrations of Hcy (10, 20 and 40 mmol/l) on the viability of HUVECs was initially detected by CCK-8 assay. The results indicated that HUVEC viability was inhibited by all the examined concentrations of Hcy, while this inhibitory effect was more notable with the increase in Hcy concentration, suggesting a concentration-dependent effect (Fig. 2).

Hcy significantly inhibits the migration ability of HUVECs. The migration ability of HUVECs is closely associated with the

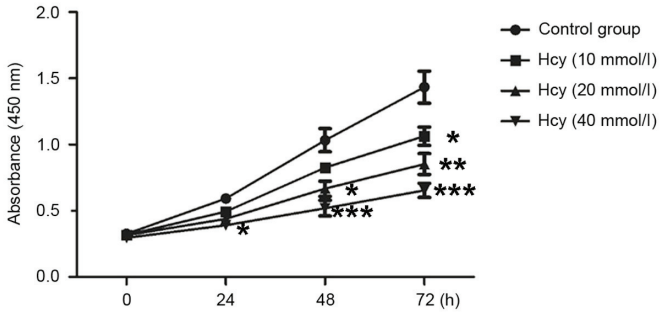

Figure 2. Effects of different concentrations of Hcy on the viability of HUVECs, determined by cell counting kit-8 assay. *P<0.05, **P<0.01 and ***P<0.001, vs. 40 mmol/l Hcy group. Hcy, homocysteine; HUVECs, human umbilical vein endothelial cells.

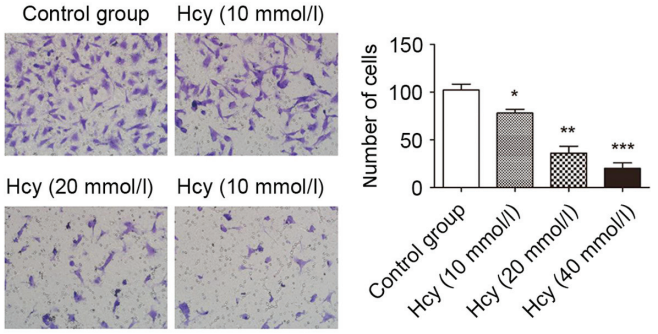

Figure 3. Effect of different concentrations of Hcy on the migration ability of HUVECs, detected by a Transwell migration assay (magnification, x200). *P<0.05, **P<0.01 and ***P<0.001, vs. control group. Hcy, homocysteine; HUVECs, human umbilical vein endothelial cells.

post-injury repair of the vascular endothelium (15,16). To determine the effect of Hcy on the migration ability of endothelial cells, HUVECs were treated with different concentrations of Hcy (10, 20 and 40 mmol/l) and then cell migration was detected by a Transwell assay. The results demonstrated that Hcy exerted a significant inhibitory effect on the migration ability of endothelial cells, and this effect was concentration-dependent (Fig. 3).

Hcy significantly downregulates VEGF expression in HUVECs. The earlier experiments of the present study preliminarily confirmed that Hcy was able to significantly inhibit the viability and migration ability of HUVECs. In order to further investigate the mechanism underlying the effect of Hcy on HUVECs, the expression levels of VEGF in HUVECs treated with Hcy was measured by RT-qPCR and western blot analysis. It was observed that treatment with Hcy significantly downregulated the mRNA (Fig. 4A) and protein (Fig. 4B and C) expression levels of VEGF in HUVECs. In addition, as determined by ELISA, the expression of VEGF-A was significantly downregulated in the supernatant of HUVECs (Fig. 4D).

Hcy inhibits the viability and migration of HUVECs by downregulating VEGF expression. Given that VEGF serves an important role in the regulation of various biological behaviors of endothelial cells, its expression level affects the viability, proliferation and migration of endothelial cells (17). Based on the earlier experimental results, Hcy was suggested to inhibit the viability and migration ability of HUVECs by downregulating VEGF expression. Thus, the study aimed to further confirm this inference. Initially, the specific siRNA of VEGF was synthesized, and transfection of cells with VEGF-siRNA did not reduce the inhibitory effect of Hcy on the viability and migration ability of HUVECs (Fig. 5A and B). This indicated that VEGF may be involved in the viability and migration ability of HUVECs. Furthermore, 5 ng/ml recombinant human VEGF was then exogenously added to HUVECs treated with Hcy. It was observed that the inhibitory effect of Hcy on the viability and migration ability of HUVECs was significantly decreased following VEGF treatment (Fig. 5C and D). This finding suggested that the inhibitory effect of Hcy on the viability and migration ability of HUVECs may be achieved by downregulating the expression of VEGF.

Discussion

DVT is a common clinical disease with high incidence, recurrence, mortality and disability rates (14). Although the pathogenesis of DVT is complex, three well-recognized causes of this disease include poor blood circulation, blood vessel wall damage and the blood hypercoagulability (18,19). In the present study, it was also determined that serum Hcy levels were significantly increased for patients with DVT. In recent years, studies have focused on the role of Hcy in the occurrence and development of DVT, as well as on the underlying mechanism (5,6). Hcy is a sulfur amino acid and an important intermediate product in the metabolism of methionine and cysteine. It is mainly produced through the demethylation of methionine in the liver, muscles and other tissues (20). Previous studies have confirmed that Hcy exerts a toxic effect on endothelial cells (8,9). In addition, the oxidation reaction of Hcy is considered to result in the production of hydrogen peroxide. Hydrogen peroxide has a strong stimulating and toxic effect on vascular endothelial cells, which may cause alterations in the vascular endothelial cell structures, thickening of the vascular wall or even blocking of the vessels (20-22). However, at present, the specific mechanism underlying the effect of Hcy on endothelial cells remains poorly understood.

In the present study, the effect of Hcy on endothelial cells was investigated. The results revealed that Hcy treatment was able to significantly inhibit the viability of HUVECs. This indicates that Hcy may cause alterations in the vessel wall structure by inhibiting the viability of endothelial cells. Following injury, the vascular endothelium experiences a complex process of neovascularization and repair. This occurs through the activation of associated growth factor receptors in vascular endothelial cells, while endothelial cells migrate and proliferate to form the surrounding matrix and embryonic structure. The embryonic structure then expands to forms a ring, which eventually forms a complete vascular cavity (11-13,23). The current study further demonstrated that Hcy had a significant inhibitory effect on the migration of endothelial cells, which may lead to inhibition of the post-injury repair process of the vascular endothelium. Taken together, the inhibitory effect of Hcy on the viability and the migratory capacity of HUVECs jointly promoted damage to the vascular wall and changes in vascular wall structure, increasing the incidence of DVT in patients with hyperhomocysteinemia.

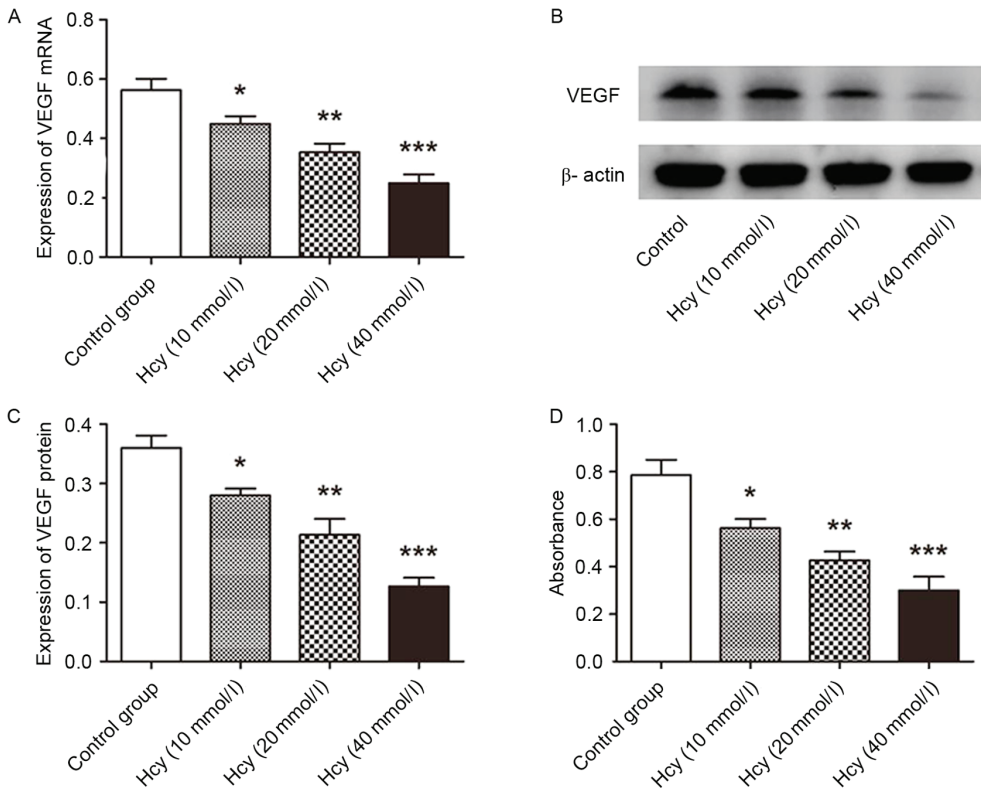

Figure 4. Hcy downregulated the expression of VEGF in HUVECs. The effect of different concentrations of Hcy on the (A) mRNA and (B) protein expression levels of VEGF in HUVECs was detected by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. (C) Quantified results of western blot analysis are shown. (D) Expression of VEGF-A in the HUVEC culture supernatant treated with different concentrations of Hcy was detected by ELISA. *P<0.05, **P<0.01 and ***P<0.001, vs. control group. Hcy, homocysteine; HUVECs, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor.

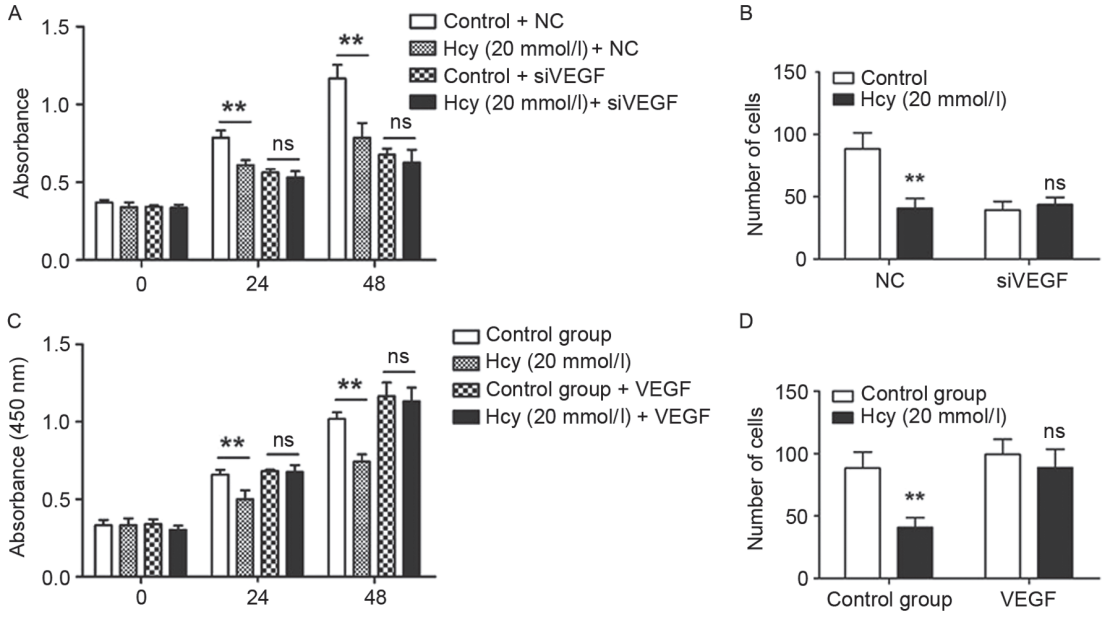

Figure 5. Hcy downregulated the expression of VEGF to inhibit the viability and migration of HUVECs. The effects of Hcy (20 mmol/l) on the (A) viability and (B) migration ability of HUVECs subsequent to VEGF expression knockdown by VEGF-siRNA were assessed by CCK-8 and Transwell migration assays, respectively. The effects of Hcy (20 mmol/l) on the (C) viability and (D) migration ability of HUVECs subsequent to addition of exogenous recombinant VEGF were examined by CCK-8 and Transwell migration assays, respectively. **P<0.01 vs. control group. Hcy, homocysteine; NC, negative control; HUVECs, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor; siRNA, small interfering RNA; CCK-8, cell counting kit-8; ns, non-significant.

Notably, the present study confirmed that Hcy inhibited the viability and migration ability of HUVECs by downregulating the expression of VEGF. VEGF is a glycosylated secreted polypeptide factor that specifically stimulates vascular endothelial

cell proliferation and angiogenesis (17). Furthermore, as a typical angiogenic factor, VEGF serves a crucial role in the regulation of the viability, proliferation and migration of endothelial cells, as well as promotes neovascularization, post-injury repair and the establishment of collateral circulation. Previous studies have confirmed that VEGF was effective in the prevention and treatment of myocardial infarction and occlusive vascular diseases, and VEGF treatment was referred to as a type of 'molecular bypass surgery' (24,25). As a vascular repair factor, the protective effect of VEGF on blood vessels in cardiovascular disease has been reported in a large number of studies (26-29). In the present study, the effect of Hcy on HUVECs was detected by upregulating and downregulating VEGF expression, which confirmed that Hcy was able to inhibit VEGF expression.

In conclusion, the present study confirmed that Hcy inhibited the viability and migration ability of endothelial cells by downregulating the expression of VEGF, causing damage to endothelial cells and affecting the post-injury repair process. This may be one of the mechanisms underlying the high incidence of DVT in patients with hyperhomocysteinemia. Furthermore, VEGF was indicated to be a key molecule in the process through which Hcy promoted DVT formation, suggesting that VEGF treatment may improve the prognosis of DVT patients with hyperhomocysteinemia. Please note that all our manuscripts now require the addition of the following sections:

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Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Authors' contributions

JY made substantial contributions to the conception and design of the work, and drafted the work. JY, LLZ, XPW, RZ, ZXM, KW, BW, HW, ZLS and GXL performed the acquisition, analysis and interpretation of data for the work. JY, LLZ, XPW, RZ, ZXM, KW, BW, HW, ZLS and GXL revised critically for important intellectual content. JY, LLZ, XPW, RZ, ZXM, KW, BW, HW, ZLS and GXL gave final approval of the version to be published. JY, LLZ, XPW, RZ, ZXM, KW, BW, HW, ZLS and GXL, agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Written informed consent was obtained from patients prior to participation, and the Ethics Committee of Qianfoshan Hospital of Shandong University (Jinan, China) provided approval for the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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