

Research Article

Role of HERC5 silencing in modulating apoptosis of EA.hy926 endothelial cells:**A Preliminary *in vitro* study with implications for deep vein thrombosis**Yiming Su^{1*}, Pimporn Thongmuang², Sarisak Soontornchai² and Guangqiu Qin³¹ Student of Doctoral Degree in Public Health Program, Graduate School, Suan Sunandha Rajabhat University, Bangkok, 10300, Thailand.² Public Health Program, Graduate School, Suan Sunandha Rajabhat University, Bangkok, 10300, Thailand³ School of Public Health and Management, Guangxi University of Chinese Medicine, Guangxi, 530200, China

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Health Science, Science and Technology Reviews. 2026;19(2):29-39.*Received: 24 January 2026; Revised: 19 May 2026; Accepted: 5 June 2026***Abstract**

Endothelial cell apoptosis contributes to a variety of vascular pathologies, including endothelial dysfunction implicated in deep vein thrombosis (DVT). HERC5, a HECT-domain E3 ubiquitin ligase tightly regulated in activated endothelial cells, has been reported to participate in inflammatory and immune responses, but its role in endothelial cell apoptosis remains insufficiently characterized. This preliminary *in vitro* study aimed to investigate the effect of HERC5 silencing on apoptosis of EA.hy926 endothelial cells. Three short hairpin RNAs targeting HERC5 (shHERC5_1, shHERC5_2, and shHERC5_3) were delivered into EA.hy926 cells by lentiviral transduction; non-targeting shRNA (shNC) and untransduced cells (Mock) served as controls. Silencing efficiency was confirmed by RT-qPCR and Western blot. Apoptosis was assessed by Annexin V/Propidium Iodide flow cytometry using shHERC5_3, which produced the greatest protein-level knockdown. Compared with shNC, all three shHERC5 constructs significantly reduced HERC5 mRNA and protein levels ($P < 0.05$). Flow cytometry showed that HERC5 silencing increased the proportion of late apoptotic cells and decreased the proportion of viable cells relative to shNC. These results provide preliminary *in vitro* evidence that HERC5 may influence endothelial cell apoptosis. Because endothelial apoptosis is one of several mechanisms associated with endothelial dysfunction and vascular pathologies including DVT, further investigation incorporating thrombosis-related endpoints and *in vivo* models is warranted to clarify whether HERC5 is mechanistically linked to DVT pathogenesis.

Keywords: HERC5, Vascular endothelial cells, Apoptosis, EA.hy926, Deep vein thrombosis

Introduction

Endothelial cell dysfunction and apoptosis are increasingly recognized as central mechanisms underlying a range of vascular and inflammatory disorders. Vascular endothelial cells (VECs) line the inner surface of blood vessels and maintain vascular homeostasis through the controlled release of vasoactive, anticoagulant, and antithrombotic mediators including nitric oxide [1], prostacyclin [2], and ectonucleotidases such as CD39 [3]. When endothelial cells undergo apoptosis, the integrity of the endothelial barrier is compromised, anticoagulant surface properties are lost, and phosphatidylserine externalization confers a procoagulant phenotype, all of which contribute to thrombosis and vascular inflammation [4,5]. Endothelial apoptosis has accordingly been implicated in atherosclerosis, hypertension-related microvascular rarefaction, diabetic vasculopathy, ischemia–reperfusion injury, and venous thromboembolic disease, indicating that endothelial apoptosis is not unique to any single condition [6–9].

HERC5 (HECT and RCC1-like domain-containing protein 5) is a 117-kDa E3 ubiquitin ligase originally identified as a Cyclin E-interacting protein [10] and subsequently characterized as a major mediator of interferon-induced ISGylation [11,12]. HERC5 is strongly upregulated in endothelial cells in response to inflammatory stimuli such as TNF- α , IL-1 β , and lipopolysaccharide (LPS) [13], suggesting tissue-specific regulation in the endothelium. Beyond its antiviral and immune functions, HERC5 has been implicated in apoptotic regulation in several cell systems: its expression is altered when tumor suppressors p53 and Rb are perturbed [10], and reduced HERC5 expression has been associated with increased metastatic potential and altered apoptotic signaling in non-small cell lung cancer [14]. However, whether HERC5 modulates apoptosis in vascular endothelial cells has not been directly established, providing the rationale for the present loss-of-function study using shRNA-mediated silencing in EA.hy926 endothelial cells.

Because endothelial apoptosis contributes to the loss of antithrombotic surface function and is one of the cellular mechanisms associated with the pathogenesis of deep vein thrombosis (DVT) [4,15], identifying molecular regulators of endothelial apoptosis may, in the longer term, provide candidate targets relevant to thrombo-inflammatory conditions. As a preliminary step toward exploring this possibility, the present study was designed to address the following narrowly defined aim: to investigate the effect of HERC5 silencing on apoptosis of EA.hy926 endothelial cells *in vitro*. We acknowledge that endothelial apoptosis is implicated in several pathological conditions and that the present study does not directly evaluate thrombosis-related endpoints. The findings are therefore intended to provide preliminary mechanistic insight into HERC5 in the endothelium, which may inform future studies that incorporate coagulation-related assays and *in vivo* DVT models.

Materials and Methods

Cell line and culture

The human endothelial cell line EA.hy926 (Kunming Cell Bank, Chinese Academy of Sciences, Kunming, China) was cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Auckland, New Zealand; Cat. No. 11965-092) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Cat. No. 10099-141) and 1% (v/v) penicillin–streptomycin solution (Solarbio, Beijing, China; Cat. No. P1400) in T25 flasks at 37 °C in a humidified atmosphere containing 5% CO₂. Medium was refreshed every 2–3 days. At 90% confluence, cells were washed twice with phosphate-buffered saline (PBS; Solarbio, Cat. No. P1020), detached with 0.25% trypsin–EDTA (Gibco, Cat. No. 25200-072) for 3 min, and subcultured at a 1:3 ratio [16].

Lentiviral vector design and transduction

Three short hairpin RNAs targeting human HERC5 (shHERC5_1: 5'-GGAGTACCCTTGGCTCAGATT-3'; shHERC5_2: 5'-GCCGCTTCCAGTGAAAGTATC-3'; shHERC5_3: 5'-GGACTGGATTACTAACATGAT-3') and a non-targeting control (shNC) were cloned into the GV248 lentiviral vector (hU6-MCS-Ubiquitin-EGFP-IRES-puromycin; Genechem, Shanghai, China) and packaged into lentiviral particles by the manufacturer using a third-generation packaging system. Untransduced EA.hy926 cells served as the Mock control. EA.hy926 cells were seeded in 6-well plates at 2×10^5 cells/well. When cells reached 40–60% confluence, lentiviral particles were added at a multiplicity of infection (MOI) of 40 in the presence of 5 µg/mL polybrene (Solarbio, Cat. No. H8761). After 12 h, medium was replaced with fresh complete DMEM, and EGFP expression was monitored by fluorescence microscopy (Olympus IX73, Tokyo, Japan) at 72 h post-transduction. Stably transduced cells were selected with 6 µg/mL puromycin (Solarbio, Cat. No. P8230) for 7 days [17].

Total RNA extraction and reverse transcription

Total RNA was extracted from stably transduced EA.hy926 cells (shNC, shHERC5_1, shHERC5_2, shHERC5_3) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA; Cat. No. 15596026) following the manufacturer's instructions. Briefly, 1 mL of TRIzol was added to each well of a 6-well plate, lysed on ice for 5 min, transferred to a 1.5-mL tube, mixed with 200 µL chloroform, vortexed for 20 s, incubated at room temperature for 5 min, and centrifuged at $12,000 \times g$ for 15 min at 4 °C. The upper aqueous phase was transferred to a fresh tube, mixed with an equal volume of isopropanol, and centrifuged at $12,000 \times g$ for 15 min at 4 °C. The pellet was washed with 75% ethanol, air-dried, and dissolved in 20 µL DEPC-treated water. RNA purity and concentration were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA); only samples with OD₂₆₀/OD₂₈₀ ratios between 1.8 and 2.0 were used. Reverse transcription was performed with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Cat. No. K1622) according to the manufacturer's instructions [18].

Quantitative real-time PCR (RT-qPCR)

RT-qPCR was carried out on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Cat. No. A25742). Primer sequences were as follows: HERC5 forward 5'-CATCACATCCTGGGGCTGGA-3', reverse 5'-TGCTGCCGACCTAAGATAAGC-3' (expected amplicon: 167 bp); GAPDH forward 5'-ATGGAAATCCCATCACCATCTT-3', reverse 5'-CGCCCCACTTGATTTTGG-3' (expected amplicon: 122 bp). Primer specificity was confirmed by *In silico* Primer-BLAST analysis (NCBI) and by melt curve analysis showing single-product amplification. Cycling conditions were: 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 60 s; followed by melt curve analysis. Relative HERC5 mRNA expression was calculated using the $2^{-\Delta\Delta C_t}$ method with GAPDH as the reference gene. Each sample was assayed in triplicate, and the experiment was performed in three independent biological replicates ($n = 3$) [19].

Western blot

Stably transduced cells were rinsed twice with ice-cold PBS and lysed in RIPA buffer (Solarbio, Cat. No. R0010) containing protease and phosphatase inhibitors (Roche, Mannheim, Germany; Cat. No. 04693116001 and 04906837001) on ice for 15 min. Lysates were centrifuged at $12,000 \times g$ for 15 min at 4 °C, and protein concentration was measured using the BCA Protein Assay Kit (Thermo Fisher Scientific, Cat. No. 23225). Equal amounts of protein (30 μ g) were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA; Cat. No. IPVH00010) at 400 mA for 50 min. Membranes were blocked in 5% non-fat milk in TBST for 1 h at room temperature and incubated overnight at 4 °C with primary antibodies against HERC5 (1:1000; Proteintech, Wuhan, China; Cat. No. 22692-1-AP) and GAPDH (1:5000; Proteintech, Cat. No. 10494-1-AP). After three washes with TBST (15 min each), membranes were incubated with HRP-conjugated goat anti-rabbit IgG (1:10000; Invitrogen, Cat. No. 31460) for 1 h at room temperature, washed again, and developed using ECL substrate (Millipore, Cat. No. WBKLS0500). Band intensity was quantified with Image Lab software v6.1.0 (Bio-Rad, Hercules, CA, USA). Three independent experiments were performed ($n = 3$) [20].

Apoptosis assay by flow cytometry

Apoptosis was assessed using the Annexin V-APC/Propidium Iodide (PI) Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA; Cat. No. 550475) according to the manufacturer's protocol. Stably transduced cells were seeded in 6-well plates and cultured to ~80% confluence. Cells were detached with EDTA-free trypsin (Solarbio, Cat. No. T1350), washed twice with ice-cold PBS, resuspended in $1 \times$ Binding Buffer at 1×10^6 cells/mL, and stained with Annexin V-APC and PI for 15–20 min at room temperature in the dark. After resuspension in 300 μ L PBS, cells were analyzed within 1 h on a BD FACSCalibur flow cytometer (BD Biosciences) and data were processed with FlowJo v10.8 (BD Biosciences). Three independent experiments were performed ($n = 3$) [21].

Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD) from three independent experiments ($n = 3$). Because the experimental design included four groups (Mock, shNC, shHERC5_1, shHERC5_2, shHERC5_3 in Western blot; shNC, shHERC5_1, shHERC5_2, shHERC5_3 in RT-qPCR), one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for multiple group comparisons. For the flow cytometry apoptosis experiment comparing shHERC5_3 with shNC, an unpaired two-tailed Student's t-test was applied. Analyses were performed with SPSS v26.0 (IBM, Armonk, NY, USA) and GraphPad Prism v8.0 (GraphPad Software, San Diego, CA, USA). A P value < 0.05 was considered statistically significant. Statistical symbols used in figures denote: *P < 0.05 , **P < 0.01 , ***P < 0.001 .

Results

Generation of HERC5-silenced EA.hy926 cells by lentiviral transduction

To investigate the role of HERC5 in endothelial cell apoptosis, EA.hy926 cells were lentivirally transduced with three independent shRNA constructs targeting HERC5 (shHERC5_1, shHERC5_2, shHERC5_3) or a non-targeting control (shNC). EGFP-positive cells were detected by fluorescence microscopy at 72 h post-transduction (Figure 1). Quantitative analysis of three independent fields per construct indicated $>90\%$ transduction efficiency for all three shHERC5 constructs and for shNC (Figure 1B). Stably transduced cells were obtained after puromycin selection (6 $\mu\text{g/mL}$, 7 days).

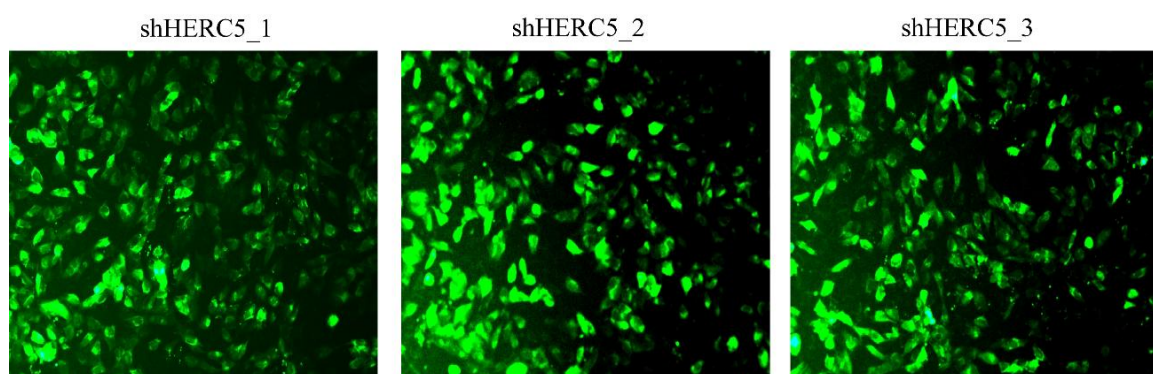


Figure 1. Lentiviral transduction efficiency in EA.hy926 cells. (A) Representative fluorescence micrographs of EGFP-positive cells 72 h after transduction with shHERC5_1, shHERC5_2, and sh HERC5_3 (scale bar = 100 μm). (B) Quantitative transduction efficiency calculated as the percentage of EGFP-positive cells relative to total cells in three randomly selected fields per construct. Data are presented as mean $\pm$ SD ($n = 3$). Untransduced (Mock) and shNC controls were included in all subsequent experiments.

HERC5 mRNA levels are reduced in shHERC5-transduced cells

RT-qPCR was performed to evaluate HERC5 mRNA silencing efficiency in stably transduced EA.hy926 cells. Compared with shNC, HERC5 mRNA levels were significantly reduced to 0.19 ± 0.03 , 0.12 ± 0.02 , and 0.27 ± 0.04 fold in shHERC5_1, shHERC5_2, and shHERC5_3 cells, respectively (one-way ANOVA followed by Tukey's post hoc test; ***P < 0.001 vs shNC for all three constructs; $n = 3$) (Figure 2).

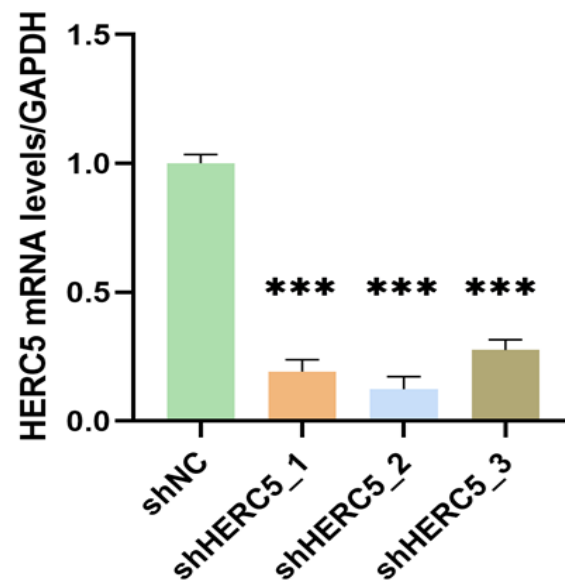
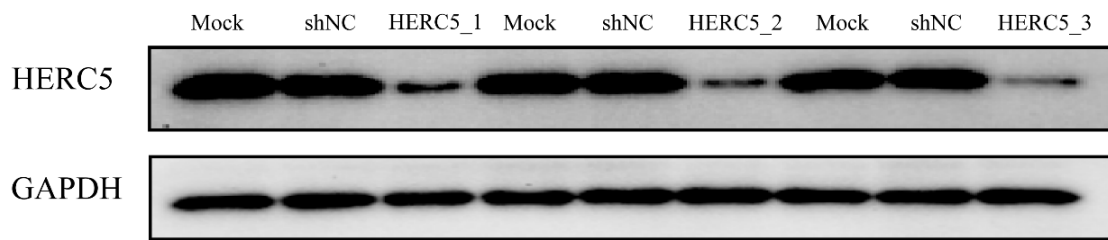


Figure 2. HERC5 mRNA silencing efficiency determined by RT-qPCR. Relative HERC5 mRNA levels in shHERC5_1, shHERC5_2, and shHERC5_3 cells were normalized to GAPDH and expressed as fold change relative to shNC. Data are presented as mean $\pm$ SD from three independent experiments ($n = 3$). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. *** $P < 0.001$ vs shNC.

HERC5 protein levels are reduced in shHERC5-transduced cells

HERC5 protein expression was assessed by Western blot in Mock, shNC, shHERC5_1, shHERC5_2, and shHERC5_3 cells (Figure 3A). Densitometric quantification, normalized to GAPDH and expressed as fold change relative to shNC, showed HERC5 protein levels of 0.50 ± 0.06 , 0.57 ± 0.09 , and 0.43 ± 0.05 in shHERC5_1, shHERC5_2, and shHERC5_3, respectively (Figure 3B). HERC5 protein levels in Mock cells were comparable to shNC (1.16 ± 0.12 fold), confirming that the non-targeting shNC did not affect endogenous HERC5 expression. All three shHERC5 constructs achieved statistically significant knockdown compared with shNC (one-way ANOVA with Tukey's post hoc test; *** $P < 0.001$ for shHERC5_1, * $P < 0.05$ for shHERC5_2, *** $P < 0.001$ for shHERC5_3; $n = 3$). Of the three constructs, shHERC5_3 produced the greatest reduction in HERC5 protein level (fold change closest to zero) and was therefore selected for subsequent functional analysis.

A



B

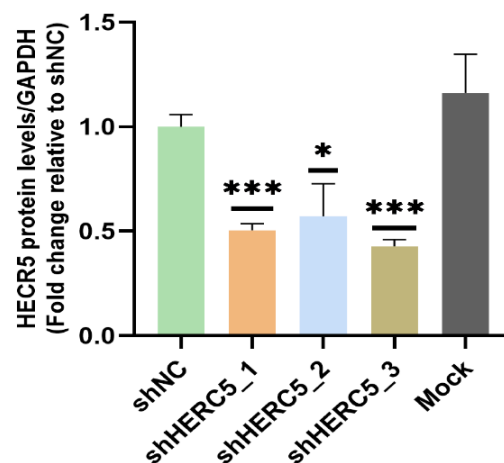


Figure 3. HERC5 protein silencing efficiency determined by Western blot. (A) Representative Western blot showing HERC5 and GAPDH protein expression in Mock, shNC, shHERC5_1, shHERC5_2, and shHERC5_3 cells. (B) Densitometric quantification of HERC5 protein levels normalized to GAPDH and expressed as fold change relative to shNC. Data are presented as mean $\pm$ SD from three independent experiments ($n = 3$). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. * $P < 0.05$, *** $P < 0.001$ vs shNC.

HERC5 silencing increases apoptosis of EA.hy926 cells

Based on the silencing efficiency at both mRNA and protein levels, shHERC5_3 was selected for the apoptosis assay. Annexin V-APC/PI staining followed by flow cytometry was used to quantify apoptotic cell populations (Figure 4). The proportion of viable cells (Annexin V $-$ /PI $-$) decreased from $91.7 \pm 2.1\%$ in shNC to $67.1 \pm 3.4\%$ in shHERC5_3 cells. Concurrently, the proportion of cells in late apoptosis (Annexin V $+$ /PI $+$) increased from $6.56 \pm 0.85\%$ in shNC to $19.8 \pm 2.6\%$ in shHERC5_3 cells. The total apoptotic rate (early plus late apoptotic cells) was significantly higher in shHERC5_3 cells than in shNC cells (unpaired two-tailed Student's t -test; ** $P < 0.01$; $n = 3$).

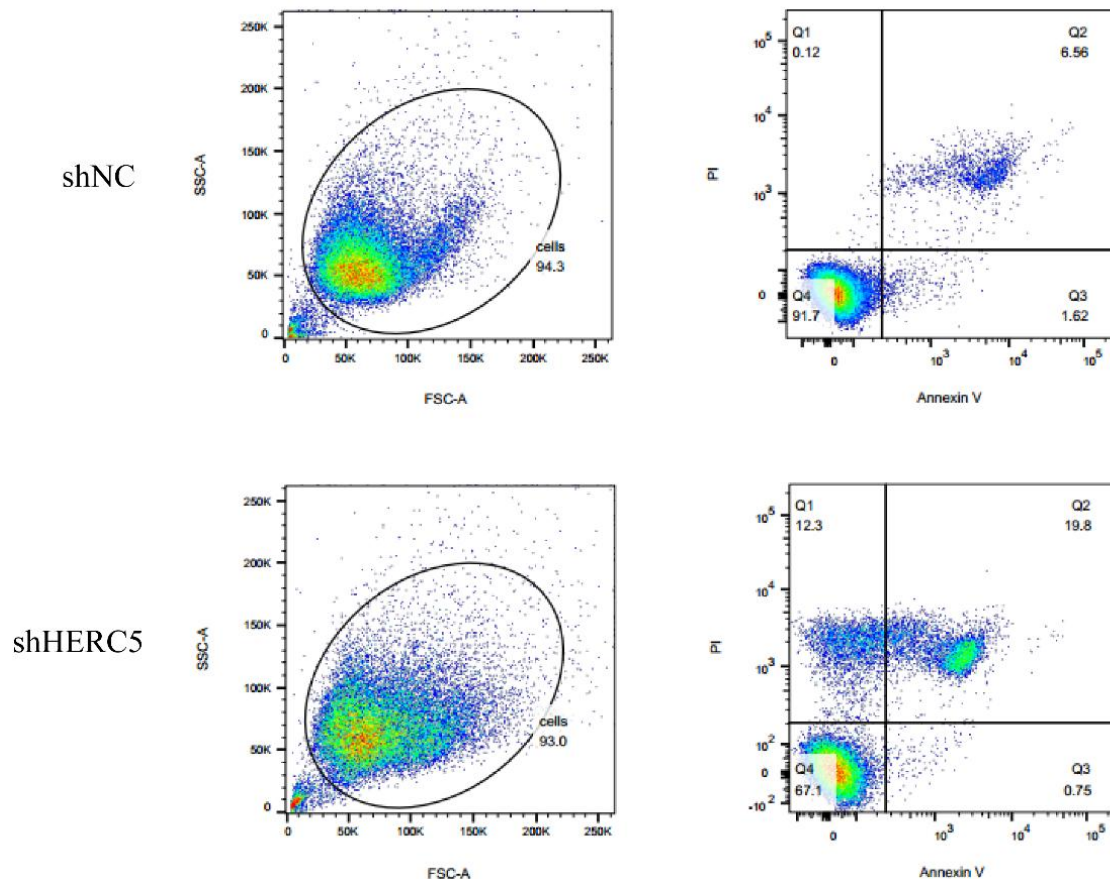


Figure 4. Apoptosis of EA.hy926 cells after HERC5 silencing. (A) Representative flow cytometry dot plots of Annexin V-APC/PI staining in shNC and shHERC5_3 cells. Q1: necrotic cells (Annexin V[−]/PI⁺); Q2: late apoptotic cells (Annexin V⁺/PI⁺); Q3: early apoptotic cells (Annexin V⁺/PI[−]); Q4: viable cells (Annexin V[−]/PI[−]). (B) Quantification of the total apoptotic rate (Q2 + Q3). Data are presented as mean $\pm$ SD from three independent experiments ($n = 3$). Statistical analysis was performed using an unpaired two-tailed Student's t-test. ** $P < 0.01$ vs shNC.

Discussion

In this preliminary *in vitro* study, we examined whether HERC5 modulates apoptosis in EA.hy926 endothelial cells. Three independent shRNA constructs reduced HERC5 mRNA and protein levels relative to a non-targeting control, and selective silencing using the most effective construct (shHERC5_3) increased the proportion of apoptotic cells, as detected by Annexin V/PI flow cytometry. These observations indicate that HERC5 may participate in the regulation of endothelial cell apoptosis *in vitro* and provide a preliminary basis for further mechanistic studies.

The EA.hy926 line, derived from the fusion of human umbilical vein endothelial cells with the A549 lung adenocarcinoma line, retains endothelial-specific features including von Willebrand factor expression and characteristic cobblestone morphology, and is widely used as a tractable model for studies of endothelial leukocyte adhesion, angiogenesis, oxidative stress responses, and apoptosis [22,23]. We acknowledge, however, that EA.hy926 cells do not fully recapitulate the heterogeneity of venous endothelium *in vivo*;

therefore, results obtained in this system should be interpreted as model-level observations requiring confirmation in primary endothelial cells and *in vivo* systems.

Several molecular mechanisms have been proposed by which HERC5 may influence apoptosis. As an interferon-inducible E3 ligase, HERC5 mediates ISGylation of multiple substrates including viral and host proteins involved in innate immune signaling [11,12]. Reported HERC5 substrates include components of the inflammatory and stress response pathways, and HERC5 has been linked to NF- κ B regulation and modulation of TNF- α /IL-1 β /LPS-induced endothelial activation [13]. Loss of HERC5 may therefore disrupt homeostatic ubiquitin/ISGylation signaling and shift the balance toward pro-apoptotic pathways. In addition, decreased HERC5 expression in non-small cell lung cancer has been associated with altered mitochondrial apoptotic signaling [14]. Whether analogous mechanisms—including ISGylation of pro-survival substrates, modulation of mitochondrial apoptotic effectors, or interaction with interferon-driven cell death programs—operate in endothelial cells remains to be determined and represents a logical direction for follow-up work.

Endothelial apoptosis has been mechanistically linked to several vascular pathologies, including but not limited to deep vein thrombosis. Apoptotic endothelial cells externalize phosphatidylserine and become procoagulant, lose anticoagulant surface molecules (thrombomodulin, heparan sulfate proteoglycans, ectonucleotidases), and display increased adhesion of leukocytes and platelets [4,15]. These changes can in principle contribute to the local milieu favoring thrombus initiation in DVT and other thrombo-inflammatory disorders [24,25]. The present study, however, does not directly demonstrate that HERC5 silencing promotes thrombus formation or modulates coagulation-related endpoints. Coagulation activation, platelet adhesion, fibrin deposition, and venous flow conditions were not assessed; nor was an *in vivo* DVT model employed. The interpretation of our findings with respect to DVT is therefore limited to a mechanistic possibility supported by the broader literature on endothelial apoptosis, rather than a directly demonstrated effect.

In summary, this study provides initial *in vitro* evidence that HERC5 contributes to the regulation of EA.hy926 endothelial cell apoptosis. The findings should be regarded as preliminary and hypothesis-generating. Confirmation in primary human endothelial cells, mechanistic dissection of HERC5-dependent ubiquitination/ISGylation targets in the endothelium, and evaluation of thrombosis-related endpoints *in vivo* will be required before a direct role for HERC5 in DVT pathogenesis can be established.

Discussion

This preliminary *in vitro* study demonstrates that lentiviral-mediated silencing of HERC5 in EA.hy926 endothelial cells increases apoptosis as assessed by Annexin V/PI flow cytometry. These findings suggest that HERC5 may participate in the regulation of endothelial cell apoptosis. Because endothelial apoptosis is one of several mechanisms that may contribute to endothelial dysfunction in DVT and related vascular pathologies, the present data support further investigation of HERC5 as a candidate modulator of endothelial apoptosis. However, this study does not directly establish a role for HERC5 in DVT pathogenesis, and additional work incorporating coagulation-related assays, primary endothelial models, and *in vivo* DVT systems will be needed to test that possibility.

Limitations and future directions

This study has several limitations. First, EA.hy926 is an immortalized hybrid cell line and may not fully reproduce the phenotype of primary venous endothelial cells; key findings should be replicated in human umbilical vein endothelial cells (HUVECs) and venous primary endothelial cells. Second, only apoptosis was examined; other endothelial functions relevant to DVT pathogenesis—such as proliferation, migration, barrier function, expression of adhesion molecules, procoagulant activity, and inflammatory mediator release—were not evaluated. Third, no direct thrombosis-related endpoints were measured, and no *in vivo* DVT model was employed; consequently, a causal link between HERC5 and DVT cannot be inferred. Fourth, the molecular mechanism by which HERC5 silencing increases apoptosis was not dissected; future work should examine candidate pathways including ISGylation of pro-survival substrates, NF- κ B signaling, and mitochondrial apoptotic effectors. Fifth, both gain- and loss-of-function approaches (HERC5 overexpression in addition to silencing) and pharmacological modulation of upstream regulators would strengthen mechanistic inference. Addressing these limitations in subsequent studies will be essential for clarifying the potential role of HERC5 in endothelial pathobiology and DVT.

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