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Mechanism of the HIF-1α/VEGF/VEGFR-2 pathway in the proliferation and apoptosis of human haemangioma endothelial cells

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Abstract

Haemangiomas (HAs) are prevalent vascular endothelial cell tumours. With respect to the possible involvement of HIF-1α in HAs, we have explored its role in haemangioma endothelial cell (HemEC) proliferation and apoptosis. shRNA HIF-1α and pcDNA3.1 HIF-α were manipulated into HemECs. HIF-α, VEGF, and VEGFR-2 mRNA and protein levels were assessed by qRT-PCR and Western blotting. Cell proliferation and viability, cell cycle and apoptosis, migration and invasion, and ability to form tubular structures were assessed by colony formation assay, CCK-8, flow cytometry, Transwell assay, and tube formation assay. Cell cycle-related protein levels, and VEGF and VEGFR-2 protein interaction were detected by Western blot and immunoprecipitation assays. An Haemangioma nude mouse model was established by subcutaneous injection of HemECs. Ki67 expression was determined by immunohistochemical staining. HIF-1α silencing suppressed HemEC neoplastic behaviour and promoted apoptosis. HIF-1α facilitated VEGF/VEGFR-2 expression and the VEGF had interacted with VEGFR-2 at protein - protein level. HIF-1α silencing arrested HemECs at G0/G1 phase, diminished Cyclin D1 protein level, and elevated p53 protein level. VEGF overexpression partially abrogated the effects of HIF-1α knockdown on inhibiting HemEC malignant behaviours. Inhibiting HIF-1α in nude mice with HAs repressed tumour growth and Ki67-positive cells. Briefly, HIF-1α regulated HemEC cell cycle through VEGF/VEGFR-2, thus promoting cell proliferation and inhibiting apoptosis.

KEYWORDS

apoptosis, cell cycle, hemangioma, hemangioma endothelial cells, HIF-1α, proliferation, VEGF, VEGFR-2

1 | INTRODUCTION

Haemangiomas (HAs) are one of the most prevalent benign tumours in young children and infants.[1] Infantile HAs are benign vascular tumours that are characterized by a distinctive life cycle consisting of rapid growth and spontaneous regression.[2] The prevalence increases with decreasing gestational age and low birthweight, and is

as high as 23% in premature babies with a birthweight of smaller than 1000 g.[3] In addition, family histories of infantile HAs, placental anomalies, and intrauterine complications including eclampsia are also principal risk factors.[4] Although most infantile HAs do not demand treatment and spontaneously regress, approximately 10%–15% of them cause complications including ulceration, obstruction, or disfigurement, and hence require treatment.[5] Haemangioma endothelial cells (HemECs) are identified as the cellular origin of infantile HAs.[6] Haemangiomas manifest with abnormal proliferation of HemECs, alter the local microenvironment and show cell transformation. There is an angiogenic factors/inhibitors imbalance, abnormal function of immune cells, and elevation of oestrogen levels.[7] Therefore, it is important to explore the mechanism of HAs from the aspect of HemEC proliferation in particular.

Various clinical observations have suggested that infantile HAs are triggered and maintained by hypoxia.[8] A group of genes are usually altered during the process that the tumour cells adapt to hypoxia, and among them, the most imperative is for the gene that encodes for hypoxia-inducible factor-1 alpha (HIF-1α).[9] HIF-1α regulates tumour cell genes and promotes the survival of tumour cells in the hypoxic microenvironment.[10] Angiogenesis is driven by vascular endothelial growth factor (VEGF) activity, which is a pro-migratory growth factor intimately linked with endothelial angiogenesis and activation.[11] HIF-1α is the main regulator for the angiogenic factors including VEGF.[12] The excessive proliferation of HemECs is caused by the increased VEGF signal via VEGF receptor-2 (VEGFR-2).[13] The VEGF-VEGFR system is an important target for anti-angiogenesis therapy in cancer.[14] In addition interference in the cell cycle[15] and thus arresting the progression of cells at the G0/G1 phase suppresses the cell proliferation of HemECs.[16] Therefore, we speculated that HIF-1α/VEGF/VEGFR-2 affected the proliferation and apoptosis of HemECs by regulating cell cycle. However, the mechanism of the HIF-1α/VEGF/VEGFR-2 pathway in regulating HemECs remains elusive. This aim of this study was to investigate the HIF-1α/VEGF/VEGFR-2 pathway in HemECs, hoping to provide reference points that will be useful in designing more effective HAs treatment.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All procedures were authorized by the laboratory animal ethics committee of Shanxi Bethune Hospital and implemented according to the Guide for the Care and Use of Laboratory Animals. All the laboratory procedures were designed to minimize the number and pain of mice.

2.2 | Cell culture and transfection

HemECs (Yaji Biotechnology) were cultured in 89% DMEM plus 10% fetal bovine serum (FBS), 1% penicillin and streptomycin in an incubator containing 5% CO_2 at 37°C. All experiments used cells in the logarithmic growth phase.

shRNA HIF-1α, pcDNA3.1 HIF-1α, pcDNA3.1 VEGF, and their negative controls shRNA NC and pcDNA3.1 NC were supplied by GenePharma. The transfection process was conducted using Lipofectamine™ 2000 (Invitrogen). The transfection concentration was 100 nM. The experiments were conducted 48 h after transfection.

2.3 | Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from HemECs and tissues with TRIzol (15596026, Invitrogen), and RNA concentration was detected by an ALLSHENG Nano-100 spectrophotometer. cDNA was synthesized with extracted RNA using the RevertAid first-strand cDNA synthesis kit (K1621, Thermo Scientific). cDNA, gene primer (Sangon), and SYBR Green I nucleic acid gel stain (S9430, Sigma-Aldrich) were mixed in the RT-qPCR system. After adding the system to the qPCR instrument (T100, Bio-Rad), 96-well plates were used for RT-qPCR: predenaturation at 95°C for 10 min, denaturation at 95°C for 15 s, annealing at 58°C for 1 min, and for a total 40 cycles. With GAPDH as the internal parameter, mRNA levels were calculated by $2^{-\Delta\Delta C_t}$ method.[17] Primer sequences are listed in Table 1.

2.4 | Western blot

The total protein of cells and tumour tissues was extracted with RIPA lysate buffer (Beyotime, Shanghai, China). About 10 μg proteins were separated on 12% SDS-PAGE and transferred to the polyvinylidene fluoride membranes. After blocking with 5% skim milk, the membranes were incubated with the primary antibodies HIF-1α (1:1000, 20,960-1-AP, 93 kDa), VEGF (1:1000, 19,003-1-AP, 46 kDa), VEGFR-2 (1:1000, 26,415-1-AP, 152 kDa), Cyclin D1 (1:5000, 26,939-1-AP, 34 kDa), p53 (1:1000, 21,891-1-AP, 44 kDa), β-actin (1:2000, 20,536-1-AP, 42 kDa), and GAPDH (1:5000, 10,494-1-AP, 36 kDa) overnight at 4°C. The next day, the membranes were exposed to the HRP-bound secondary antibody for 2 h, and the signals were

TABLE 1 RT-qPCR primer sequences.

	Forward (5′-3′)	Reverse (5′-3′)
HIF-1α	GAACGTCGAAAAGAAAA GTCTCG	CCTTATCAAGATGCGAAC TCACA
VEGF	GGGCAGAATCATCACGAAGT	AAATGCTTTCTCCGCTCTGA
VEGFR-2	CAAGTGGCTAAGGGCATGGA	ATTTCAAAGGGAGG CGAGCA
GAPDH	GGAGCGAGATCCCTCCA AAAT	GGCTGTTGTCATACTTC TCATGG

detected using an enhanced chemiluminescence kit (Bio-Rad). With GAPDH as the internal parameter, semiquantitative analysis was conducted using the ImageJ software (NIH). Antibodies were all purchased from Proteintech Group, Inc.

2.5 | Colony-forming assay

The 200 HemECs per group were transferred to a culture dish containing 10 mL of complete medium. The plates were slowly shaken so that HemECs were distributed evenly. After 14 days of conventional incubation, HemECs in culture dishes were collected. During the incubation, the medium was refreshed every 2 days. Collected HemECs were stained with Giemsa for 20 min. Finally, the number of HemECs in the visual field was counted using a Nikon microscope (E100) to calculate the relative colony formation rate.

2.6 | Cell counting kit-8 (CCK-8)

A single cell HemECs suspension (10^5 cells/mL) was seeded into 96-well plates. HemECs were incubated for 24, 48, and 72 h under normal conditions and 10 μL CCK-8 solution (HY-K0301, MedChemExpress) was added to each well. The mixture was incubated for another 4 h to fully combine CCK-8 with mitochondria in HemECs. Finally, functional detection was conducted using a SynergyTM LX multifunctional microplate detector (BioTek). By analysing the optical value at 450 nm, the viability of HemECs was detected.

2.7 | Apoptosis analysis

HemECs (1×10^5 clones) were trypsinized, washed with cold PBS, and resuspended in the binding buffer as the apoptosis kit (V13242, Thermo Fisher Scientific) instructed. The fixed cells were added with FITC-AnnexinV and propidium iodide (PI) in the dark for 20 min. Then, Annexin V binding buffer was added to the mixture before fluorescence was measured using the CytoFLEX St flow cytometry (Beckman Coulter). Cell Quest software (Becton Dickinson) was applied for apoptosis analysis.

2.8 | Cell migration and invasion assays

About 10^4 HemECs per well in six-well plates were cultured for 24 h, and cell monolayers were scratched with a 100-μL micropipette tip. After the debris cells and floating cells were rinsed with PBS, the migration of cells was observed and imaged at 0 and 48 h under a light microscope (Olympus).

Cell invasion was determined using 8-mm pore-size Transwell filters (Costar, Corning Incorporated).[18] In short, 10^4 cells/well in 100 μL FBS-free DMEM were placed on the apical chamber of Transwell in the 24-well plates, and the basolateral chamber was filled with 300 μL DMEM containing 10% FBS. After 24-h incubation, HemECs invading the lower surface of the filter were stained with crystal violet, counted, and imaged under a light microscope.

2.9 | Endothelial tube formation assay

Before transfection, HemECs seeded in 96-well plates were cultured at 37°C containing 5% CO_2 for 6 h. Matrigel (BD Biosciences) and ECM were mixed at 1:2, and 50 μL of mixture was put in each well, incubated at 37°C for 20 min to solidify Matrigel. Tube formation was imaged under a microscope and quantified using the Image J software.

2.10 | Immunocoprecipitation

The 4×10^7 cells were lysed with 1 mL RIPA for 30 min and centrifuged at 4°C at 12000×*g* for 30 min. The 10 μL supernatant was collected and added in 2×SDS loading buffer solution and denatured at 99°C for 10 min. The pretreated protein A/G beads (Bimake) and 300 μL RIPA were added to 6 μL antibody and incubated for 15 min at 4°C. The mixture was placed on the magnet for 1 min, the

supernatant was removed, and the samples were washed three times. The beads were resuspended in the sample (300 μL), incubated at 4°C overnight, and gently rotated. Then, the mixture was placed on the magnet for 1 min, the supernatant was removed, and the samples were washed three times. The beads were added to 1×SDS loading buffer, denatured for 10 min at 99°C, and centrifuged for 10 min at 12000×*g*, with the supernatant preserved and evaluated by Western blot.

2.11 | Cell cycle analysis

Cell cycle distribution was analysed by flow cytometry (Beckman Coulter). The treated cells were detached with trypsin, centrifuged for 5 min at 1000×*g*, and washed with cold PBS. Next, the cell mass was resuspended and fixed overnight with 70% cold ethanol. After washing again with PBS, the cell precipitation was resuspended in the staining solution containing PI (50 μg/mL), Dnase-free RNase (100 μg/mL), and Triton-100 (0.3%, Bioengineering Corporation). Finally, cells were incubated at 37°C in the dark for 30 min. The fraction of cell population at each stage of the cell cycle was determined as a function of the DNA content analysed by flow cytometry.

2.12 | In vivo experiment

BALB/c nude mice (male, 5 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd (SYXK [Shanghai] 2022–0012). Subsequently, 100 μL PBS containing 5×10^6 shRNA HIF-1α or shRNA NC manipulated HemECs were injected subcutaneously into the BALB/c nude mice, with six mice in each group. According to the formula: (long×width^2)/2, tumour volume was evaluated every week for 5 weeks. On the 35th day, mice were euthanized by intraperitoneal injection of excessive sodium pentobarbital (800 mg/kg), and the tumours were collected and weighed.

2.13 | Immunohistochemical (IHC) analysis

The tumours from animal models were fixed in 4% formalin, embedded in paraffin, and cut into 3-μm sections. The sections were incubated with anti-Ki67 antibody (1:500, 27309-1-AP, Proteintech Group) overnight at 4°C and then with a secondary antibody working solution (Dako, GK500705) for 2 h. Ki-67 expression in tumour tissues was observed using diaminobenzidine substrate kit (Invitrogen).

2.14 | Statistical analysis

SPSS 21.0 (IBM Corp.) was used for data analysis. Kolmogorov–Smirnov demonstrated that the data were in normal distribution. Data were depicted as mean ± standard deviation. Data among groups were compared by one-way ANOVA, followed by Tukey's test. *p* was obtained by a bilateral test. $p<.05$ indicated statistical significance.

3 | RESULTS

3.1 | HIF-1α promoted HemEC malignant behaviours

Hypoxia-inducible factors are a principal factor in tumour progression.[19,20] To study the role of HIF-1α in HemEC growth, shRNA HIF-1α/NC and pcDNA3.1 HIF-1α/NC were introduced into HemECs respectively. HIF-1α levels in shRNA HIF-1α-transfected cells were diminished, and HIF-1α levels in pcDNA3.1 HIF-1α transfected cells were elevated, indicating successful transfections (Figure 1A,B, all $p<.01$). Colony formation assay and CCK-8 revealed that HIF-1α silencing inhibited HemEC proliferation and viability, while HIF-1α upregulation brought the opposite results (Figure 1C,D; all $p<.01$). Flow cytometry demonstrated that HIF-1α enhanced apoptosis, while HIF-1α upregulation repressed apoptosis (Figure 1E, all $p<.01$). Transwell assays showed that HIF-1α silencing suppressed HemEC migration and invasion, while HIF-1α overexpression promoted migration and invasion (Figure 1F, $p<.05$). In addition, the effect of HIF-1α on endothelial tube formation capacity was investigated. After HIF-1α knockdown, HemEC complex branching tube network structure became simpler, while HIF-1α elevation facilitated the endothelial tube formation (Figure 1G, all $p<.001$). The results suggested that HIF-1α stimulated more aggressive HemEC behaviour.

3.2 | HIF-1α augmented VEGF/VEGFR-2 expression

To study the relationship between HIF-1α and VEGF/VEGFR2, the VEGF/VEGFR2 pathway expression was determined after HIF-1α knockdown. RT-qPCR and Western blot demonstrated that after HIF-1α knockdown, VEGF

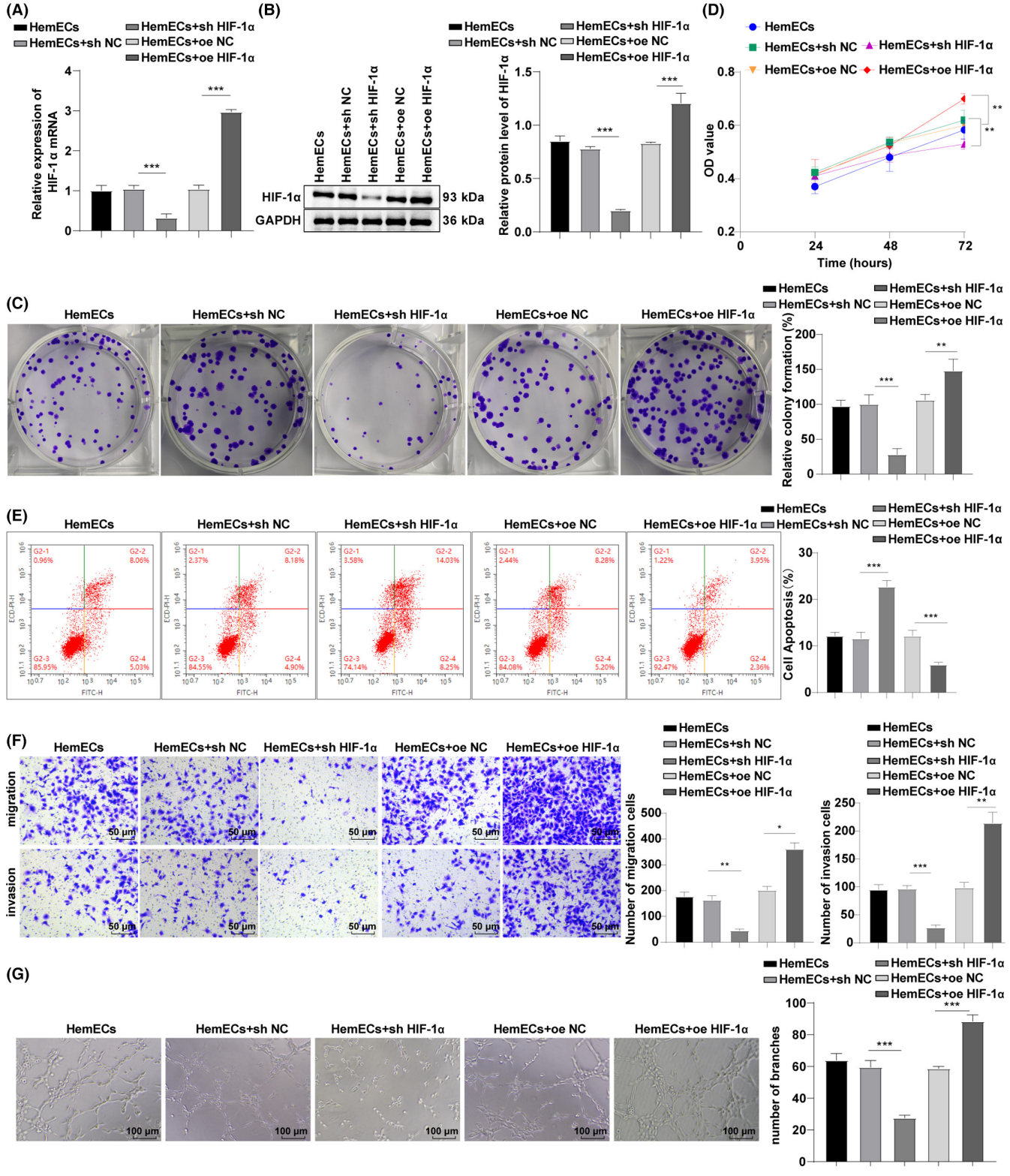

FIGURE 1 HIF-1α promoted HemEC neoplastic behaviour HemECs were manipulated with 100 nM nM shRNA HIF-1α/NC or 100 nM pcDNA3.1 HIF-1α/NC. (A) HIF-1α mRNA expression was assessed by RT-qPCR; (B) HIF-1α protein level was determined by Western blot; (C) Cell proliferation was detected by colony formation assay; (D) Cell viability was detected by CCK-8; (E) Apoptosis was detected by flow cytometry; (F) Cell migration and invasion abilities were detected by Transwell assays; (G) Cell tube formation was detected by endothelial tube formation assay. Cell experiment was repeated three times. Data were expressed as mean ± standard deviation. One-way ANOVA was employed for comparisons among groups, followed by Tukey's multiple comparisons test. *$p < .05$, **$p < .01$, ***$p < .001$.

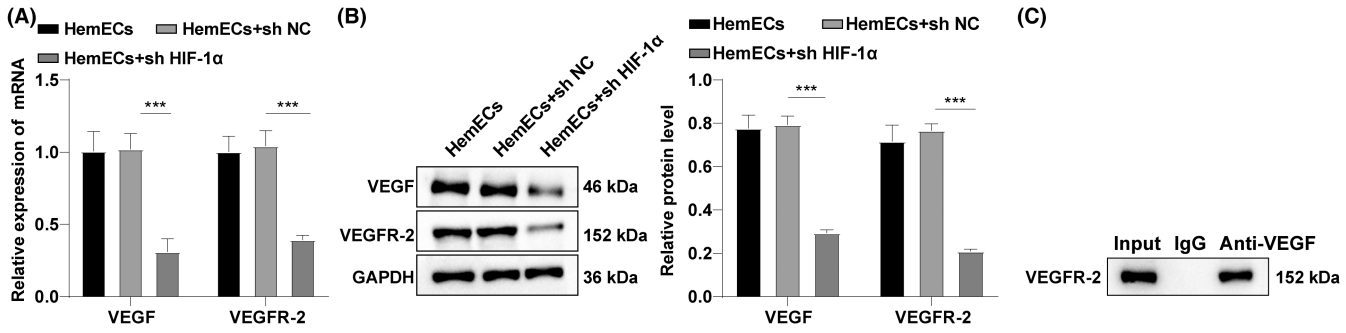

FIGURE 2 HIF-1α promoted VEGF/VEGFR-2 expression. (A) VEGF and VEGFR-2 mRNA levels were assessed by RT-qPCR; (B) VEGF and VEGFR-2 protein levels were assessed by Western blot; (C) The interaction between VEGF and VEGFR-2 was detected by immunoprecipitation. Cell experiment was repeated three times. Data were expressed as mean ± standard deviation. One-way ANOVA was applied for comparisons among groups, followed by Tukey's multiple comparisons test. ***$p < .001$.

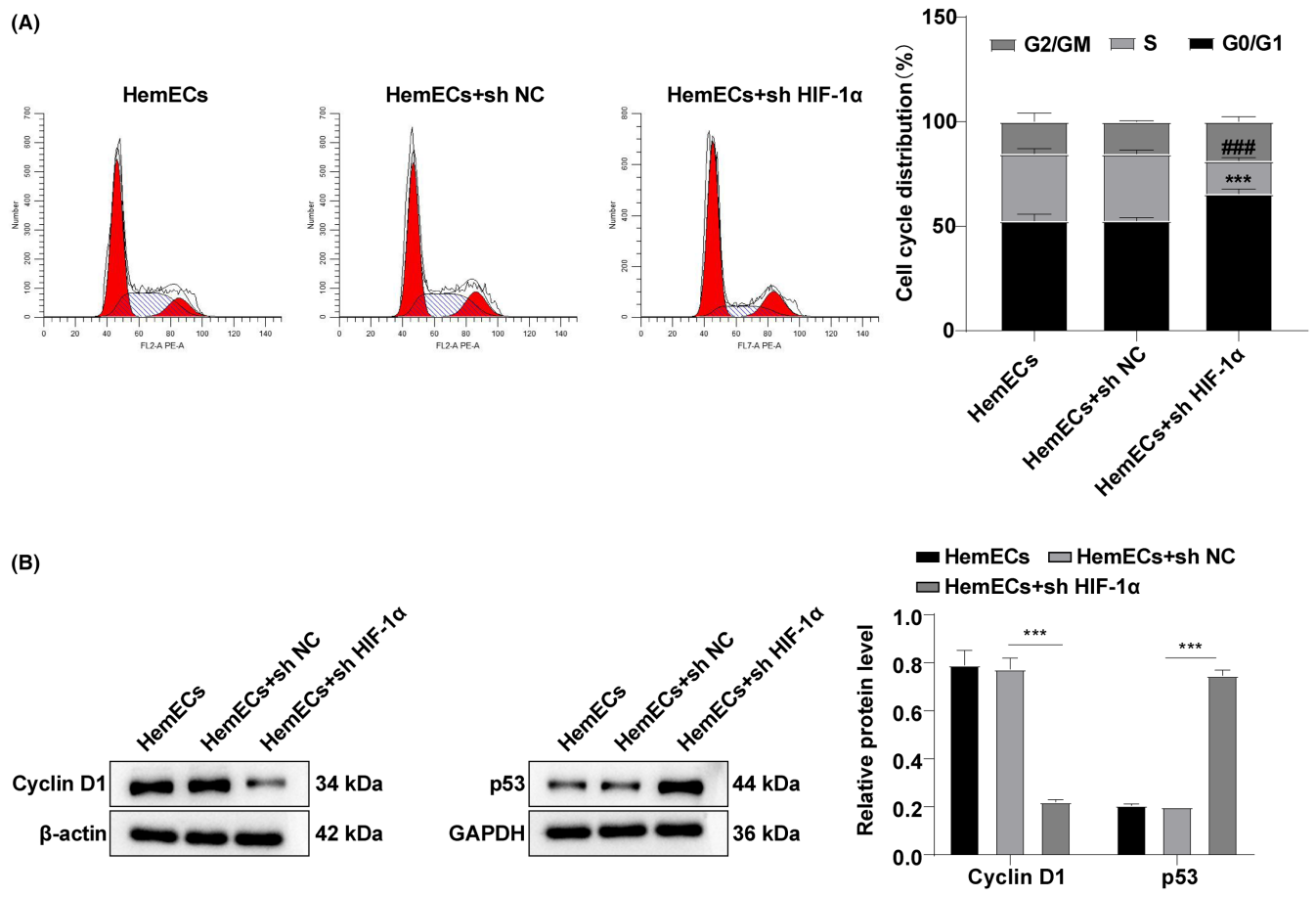

FIGURE 3 HIF-1α arrested HemECs at G0/G1 phase. (A) Cell cycle was detected by flow cytometry; (B) Cell cycle-related protein levels were determined by Western blot. Cell experiment was repeated three times. Data were expressed as mean ± standard deviation. One-way ANOVA was adopted for comparisons among groups, followed by Tukey's test. ###$p < .001$, ***$p < .001$.

and VEGFR-2 levels were also diminished (Figure 2A,B, all $p < .01$). VEGF regulates endothelial cells through interaction with VEGFR-2.[21] Immunoprecipitation assay confirmed that VEGFR-2 could be precipitated by VEGF antibody (Figure 2C), indicating that there was interaction between VEGF and VEGFR in HemECs. Shortly, HIF-1α promoted VEGF/VEGFR-2 levels.

3.3 | HIF-1α knockdown caused G0/G1 phase arrest of HemECs

The effect of HIF-1α on the cell cycle of HemECs was studied further. Flow cytometry results in Figure 3A revealed that HIF-1α knockdown increased the number of cells arrested in G0/G1 phase, and decreased the number

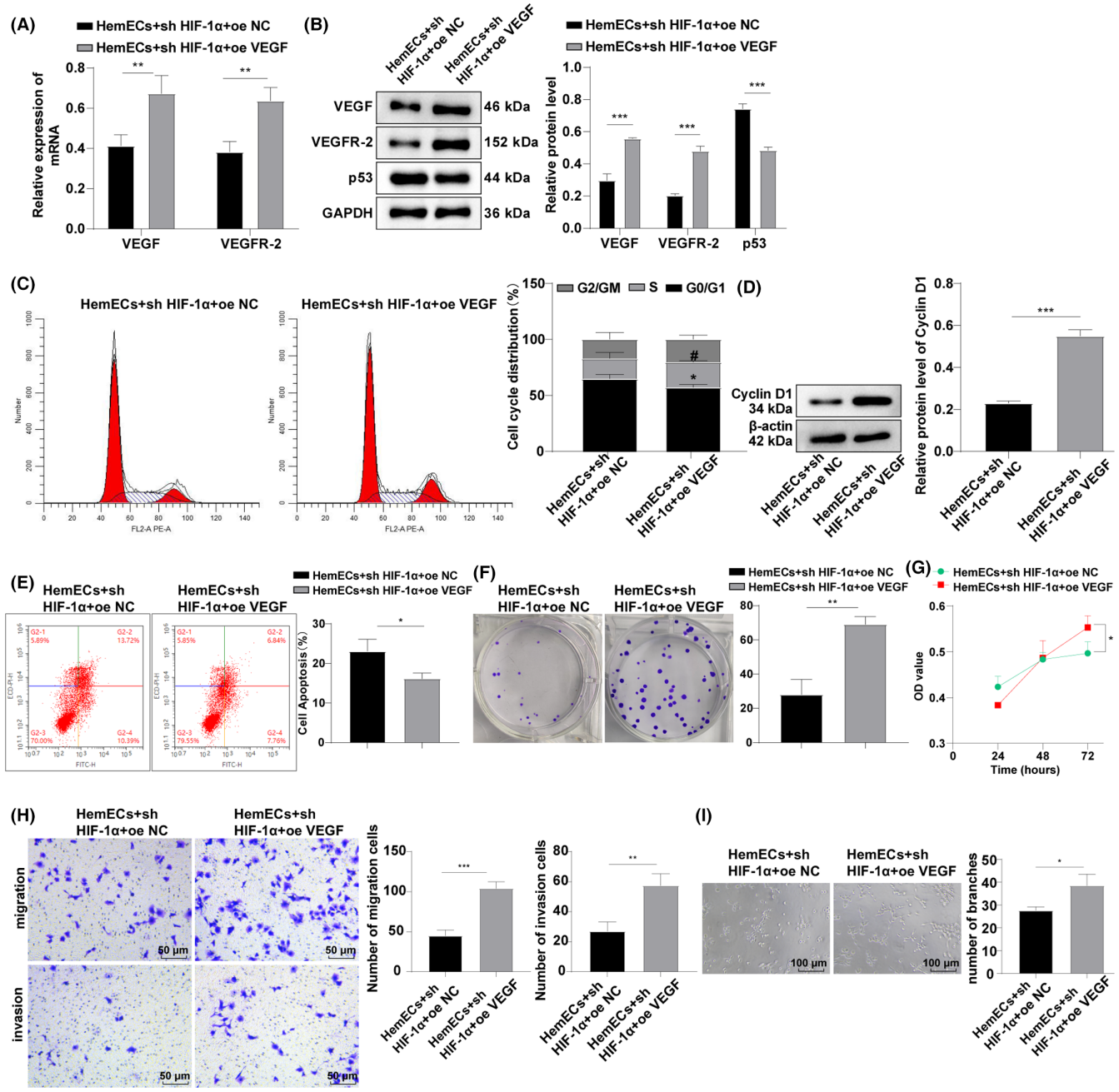

FIGURE 4 VEGF overexpression partially annulled the effects of HIF-1α silencing on suppressing HemEC malignant behaviours. HemECs were transfected with 100 nM shRNA HIF-1α and 100 nM pcDNA3.1 VEGF/NC. (A) VEGF and VEGFR-2 mRNA levels were assessed by RT-qPCR; (B) VEGF, VEGFR-2 and p53 protein levels were determined by Western blot; (C) Cell cycle was detected by flow cytometry; (D) cyclin D1 expression was assessed by Western blot; (E) Apoptosis was detected by flow cytometry; (F) Cell proliferation was detected by colony formation assay; (G) Cell viability was detected by CCK-8; (H) Cell migration and invasion abilities were detected by Transwell assays; (I) Cell tube formation was detected by endothelial tube formation assay. Cell experiment was repeated three times. Data were expressed as mean ± standard deviation. Independent sample *t*-test was applied for comparison between two groups. #$p < .05$, *$p < .05$, **$p < .01$, ***$p < .001$.

of cells in S phase (all $p < .001$). Cyclin D1 is overexpressed in many tumours, including sporadic hemangioblastoma, and plays a key role in the control of cell cycle.[22] The activation of p53 tumour suppressor leads to cell cycle arrest.[23] Western blot showed that HIF-1α knockdown repressed cyclin D1 protein level and stimulated p53 protein level (Figure 3B, all $p < .01$). To conclude, HIF-1α silencing resulted in HemEC arrest at G0/G1 phase.

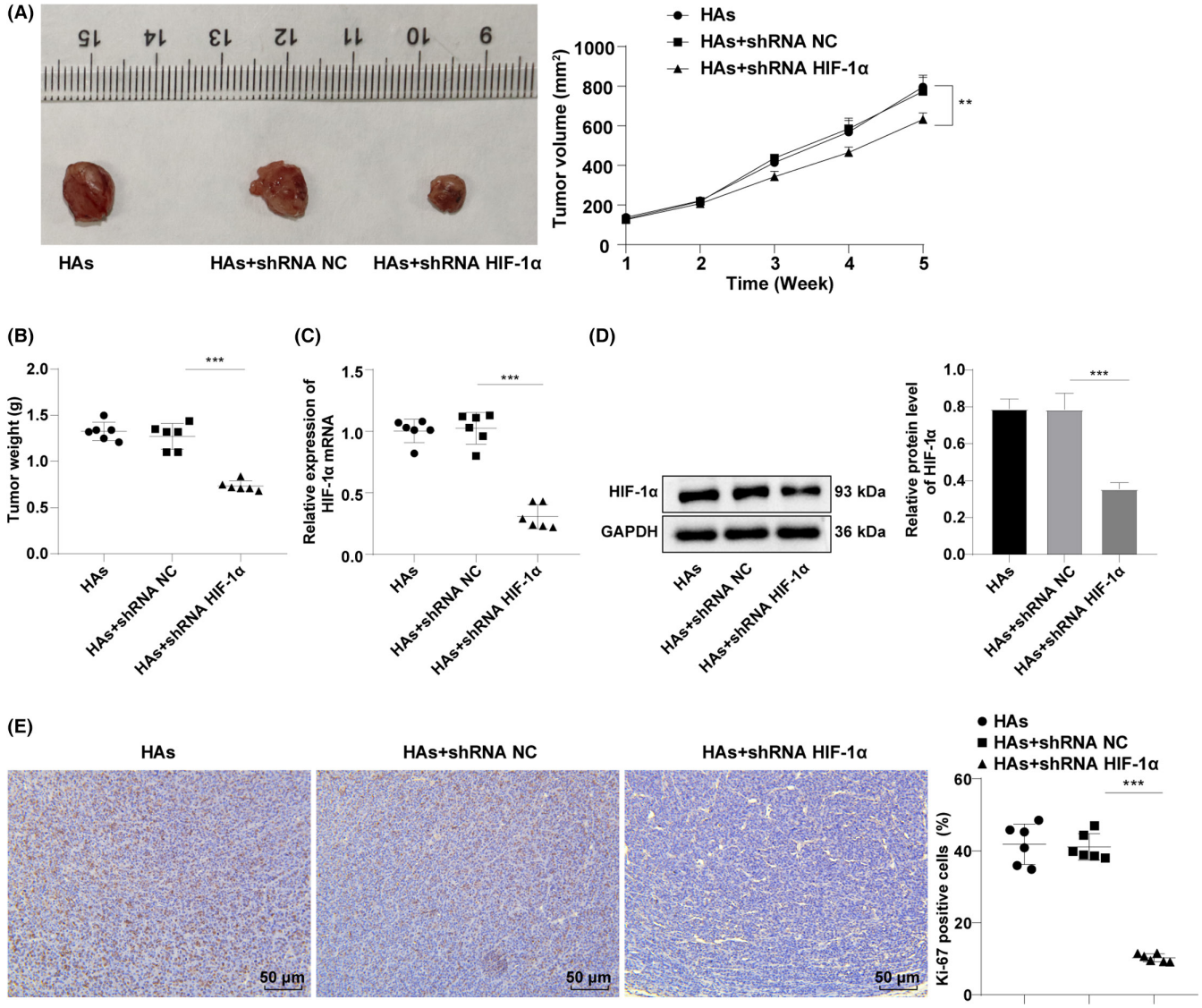

FIGURE 5 Knockdown of HIF-1α limited tumour progression in nude mice with HAs. Tumour models in vivo were established by subcutaneous injection of 5×10^6 HemECs (transfected with shRNA HIF-1α/NC) in nude mice. (A) Tumour volume; (B) Tumour weight; (C) HIF-1α expression in tumour tissues was assessed by RT-qPCR; (D) HIF-1α protein level in tumour tissues was determined by Western blot; (E) Ki-67 expression was assessed by IHC. $N=6$. Data were expressed as mean ± standard deviation. One-way ANOVA was employed for comparisons among groups, followed by Tukey's multiple comparisons test. $^{**}p<.01$, $^{***}p<.001$.

3.4 | VEGF overexpression partially averted the inhibitory effects of HIF-1α knockdown on HemEC malignant behaviours

To ascertain that HIF-1α regulated HemEC neoplastic behaviour through VEGF/VEGFR-2, a functional rescue experiment was conducted by silencing HIF-1α in HemECs and transfecting pcDNA3.1 VEGF simultaneously. RT-qPCR and Western blot showed that after pcDNA3.1 VEGF transfection, VEGF, and VEGFR-2 levels in HemECs were raised (Figure 4A,B, all $p<.01$), indicating successful transfection. Flow cytometry showed that VEGF overexpression reduced the G0/G1 phase arrest of HemECs, inhibited cell apoptosis (Figure 4C,E, all $p<.05$), raised cyclin D1 protein level, and repressed p53 levels (Figure 4B–D, all $p<.001$). Additionally, VEGF overexpression increased cell proliferation, viability, migration, invasion, and tubular formation (Figure 4F–I, all $p<.05$). Overall, VEGF overexpression partially abrogated the effects of HIF-1α knockdown on inhibiting HemEC malignant behaviours.

3.5 | HIF-1α knockdown suppressed tumour progression in nude mice with HAs

To elucidate the carcinogenic effect of HIF-1α in HAs progression, animal models were established by subcutaneous

inoculation of 5×10^6 shRNA HIF-1α or shRNA NC transfected HemECs. Compared with the shRNA NC group, HIF-1α knockdown blocked the growth of tumours, manifesting in the reduction in tumour volume and weight in mice (Figure 5A,B, all $p<.01$). RT-qPCR and Western blot demonstrated lower HIF-1α expression in tumour tissues in the HAs + shRNA HIF-1α group than in the HAs + shRNA NC group (Figure 5C,D, $p<.01$). In addition, IHC staining showed that HIF-1α silencing reduced Ki-67-positive cells in tumour tissues (Figure 5E, $p<.01$). Briefly, HIF-1α knockdown repressed tumour progression in nude mice with HAs.

4 | DISCUSSION

Haemangiomas are common vascular endothelial cell tumours, which start as benign cases of infant HAs and can progress to low-graded malignant hemangioendothelioma and angiosarcoma.[24] In addition to the predominantly harmless vascular skin changes, they should be treated due to the potentially threatening implications.[5] Evidence has shown that HIF-1α functions as a strong pro-angiogenic molecule that facilitates the proliferation of endothelial cells in infant HAs.[25] This study found that HIF-1α modulated the cell cycle of HemECs through VEGF/VEGFR-2, thus stimulating the proliferation of HemECs and repressing their apoptosis.

Hypoxia is a key stimulator of endothelial migration, apoptosis sensitivity, and angiogenesis in tumours.[26] HIF-1a is an essential regulator in the angiogenesis induced by hypoxia, which is a main pro–angiogenic factor in various hypoxic solid tumours, and overexpression of HIF-1α abrogates VEGF expression and HemEC growth.[12] Our findings revealed that after knockdown of HIF-1α, the viability, proliferation, invasion, migration, and endothelial tubulogenesis of HemECs were all suppressed, and apoptosis was intensified, while these trends were annulled by overexpression of HIF-1α. Likewise, repression of the HIF-1a-VEGF axis leads to the antiproliferative property of curcumin in HemECs.[27] Silencing of HIF-1a limits the viability and migration rate of HemECs.[28] Propranolol blocks the proliferation, tube formation, and migration of HA cells via the HIF-1α dependent mechanisms.[29] Thus HIF-1α amplified the more aggressive behaviour of HemECs. Subsequently, we conducted in vivo experiments and found that after HIF-1α silencing, tumour growth was limited, and Ki-67-positive cells were reduced, which manifested that knockdown of HIF-1α blocked the progression of HAs in nude mice.

Cyclin D1 is a principal regulator for the cell cycle with an essential role in cancer pathogenesis and in determining uncontrolled cellular proliferation. Its expression in normal cells is strictly regulated, and conversely it is intensified in cancer in various ways.[30] The p53 tumour suppressor activation causes cell cycle arrest.[23] Our results showed an increase in cells blocked in G0/G1 phase, and decrease in cells in S phase after knockdown of HIF-1α. Silencing of HIF-1α diminished Cyclin D1 and elevated p53 levels. Knockdown of HIF-1α induces cell cycle arrest in G0/G1 phase in oesophageal squamous cell carcinoma.[31] Lentiviral shRNA targeting HIF-1α causes G0/G1-phase cell cycle arrest in glioma.[32] Collectively, HIF-1α knockdown caused the arrest of HemECs at G0/G1 phase.

Haemangiomas are the tumours formed by the hyperproliferation of HemECs that resulted from promoted VEGF signalling via VEGFR2, and a potential mechanism involved in the formation of HAs is the VEGF/VEGFR2 pathway alteration in endothelial cells.[13] HemECs express low levels of VEGFR-1 and phosphorylated VEGFR-2 constitutively in contrast to normal endothelial cells from newborn foreskin.[33] We knocked down HIF-1α and discovered that knockdown of HIF-1α suppressed the levels of VEGF and VEGFR2 and VEGF that could interact with VEGFR in HemECs. The VEGF to VEGFR2 binding causes autophosphorylation of specific tyrosine residues in the VEGFR2 cytoplasmic domain, and inhibition of VEGF/VEGFR2 pathway is a potent to hinder tumour growth.[34] Notably, VEGF binds to VEGFR2 on ECs, thus activating key angiogenic pathways, such as ERK1/2, STAT3, AKT, which facilitate EC growth.[34–36] Altogether, HIF-1α stimulated the expression of VEGF/VEGFR-2.

Furthermore, we conducted functional rescue assays by silencing HIF-1α and introducing pcDNA3.1 VEGF into HemECs simultaneously and elaborated that overexpression of VEGF decreased HemECs arrest at the G0/G1 phase, repressed the apoptosis, and facilitated the viability, proliferation, invasion, migration, and tubular formation of HemECs. Overexpression of VEGFA alone promotes the invasion and proliferation of HemECs but reduces apoptosis.[37] Deletion of VEGF can abolish IL-6-induced HA progression.[38] VEGF induces VEGF-dependent activation of VEGFR2 to facilitate HemEC growth.[39] Targeted inhibition of VEGF can suppress the proliferation of HA cells.[35,36] Briefly, overexpression of VEGF partially averted the properties of HIF-1α silencing in repressing the aggressive behaviour of HemECs. In summary, this study supported that HIF-1α silencing might be a new target for the treatment of HAs, which regulated the endothelial cell cycle through VEGF/VEGFR-2, thus inhibiting cell malignant behaviours. However, the intracellular transduction pathway of VEGFR-2 in HemECs has not been fully studied. The results of VEGF-A stimulating different types of endothelial cells in vitro manifest that the VEGFR-2 signal

transduction depends on the downstream effects of ERK,[40–42] and the possible downstream mechanisms need to be explored in future research.

AUTHOR CONTRIBUTIONS

WPZ is the guarantor of integrity of the entire study and contributed to the study design, study concepts, and manuscript editing; LS contributed to the literature research, data acquisition, and data analysis; HXG contributed to the definition of intellectual content, statistical analysis, and experimental studies; SQW contributed to the manuscript preparation, experimental studies, and manuscript review; All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

All authors declare that there is no conflict of interests in this study.

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