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Diosgenin inhibits tumor angiogenesis through regulating GRP78-mediated HIF-1α and VEGF/VEGFR signaling pathways

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The present paper describes the molecular mechanism of diosgenin on tumor microenvironment angiogenesis and the regulation of GRP78 in angiogenesis signaling pathway. CCK8 method was used to evaluate the effect of different concentrations of diosgenin on HUVEC activity in hypoxic microenvironment. Apoptosis was detected by Annexin V/PI staining. Tube Formation experiment was conducted to evaluate angiogenesis. Western Blot assay was applied to detect the expressions and phosphorylation levels of the angiogenesis-related pathways HIF-1α, GRP78, VEGF/VEGFR, PI3K/AKT, ERK, and FAK in rheumatoid HUVEC. Silencing GRP78 by siRNA interference technology was employed to investigate the mechanism of GRP78 involved in the regulation of angiogenesis. The results indicated that diosgenin can significantly inhibit the cell viability of hypoxic HUVEC and the significance is dependent on drug concentration. As the concentration increases, HUVEC activity decreases. Cell apoptosis is induced in a dose-dependent manner and angiogenesis can be significantly inhibited. The hypoxic microenvironment can significantly increase the expressions of HIF-lα, GRP78, VEGF/VEGFR, PI3K/AKT, ERK, FAK proteins in angiogenesis-related pathways, and can also enhance the phosphorylation of AKT, ERK1/2 and FAK proteins, which can be decreased by drug intervention. After silencing GRP78, the angiogenesis-related pathway proteins HIF-lα and VEGF/VEGFR are significantly reduced, thus inhibiting the activation of AKT, ERK1/2, and FAK. The anti-tumor angiogenesis mechanism of diosgenin inhibiting the expression of HIF-lα, GRP78, VEGF/VEGFR, PI3K/AKT, ERK1/2 and FAK signaling pathways may be through multiple pathways and targets, and GRP78 is involved in the regulation of angiogenesis signaling pathway.

1. Introduction

Angiogenesis is one of the main factors of tumor growth, metastasis and invasion. It provides nutrient and waste excretion pathways for tumors, and also pathways for tumor cells to enter the circulatory system. It is a key step and necessary condition for tumor development and metastasis. Therefore, inhibiting tumor angiogenesis is an important way to inhibit tumor growth and metastasis. Multiple cytokines and signaling pathways are involved in the regulation and mediation of angiogenesis (Chen et al. 2016; Samant and Shevde 2011; Dong et al. 2011).

VEGF-mediated signaling cascades participate in every process of angiogenesis and including the occurrence and development, making it the most important regulator of angiogenesis and differentiation. VEGF binds to the corresponding receptor VEGFR to play a biological role, triggering downstream signals cascading signaling transduction pathways, including the ERK, PI3K/AKT, and FAK, etc. VEGF can induce endothelial cell division, proliferation and migration by promoting endothelial cell sprouting, thus accelerating the degradation of vascular basement membrane, promoting the formation of the foot ring, reinforcing the activity of endothelial cells, promoting angiogenesis, and accelerating tumor growth and invasion (Takahashi et al. 2016; Mackay et al. 2014; Scartozzi et al. 2016; Paszek et al. 2014; Quittet et al. 2015).

Glucose-regulated protein 78 (GRP78) is a member of the heat shock protein 70 family. It is significantly elevated in endoplasmic reticulum stress and therefore is considered to be a biomarker of endoplasmic reticulum stress. Hypoxia can increase the expression of GRP78 in various tumor cells and endothelial cells. Recently more and more studies have found that GRP78 is closely related to tumor neovascularization (Cheng et al. 2014; Hongo et al. 2013). Therefore, the anti-tumor mechanism, featuring inhibiting angiogenesis, tumor growth and invasion by improving tumor microenvironment, has important significance for the prevention and control of tumor growth and metastasis.

Diosgenin is the main component of the traditional Chinese medicine *Paris polyphylla*. It has certain effects on rheumatism, cardiovascular disease, lymphoblastic leukemia, cellular encephalitis, skin diseases, tumors, etc. However, it is unclear how exactly diosgenin is inhibiting angiogenesis and tumor growth by improving the tumor microenvironment. The present study was done to investigate whether diosgenin has an effect on angiogenesis, so as to provide a theoretical basis for its clinical application.

Table: Effect of $CoCl_2$-induced hypoxia on cell viability

$CoCl_2$ (μM)	24 h	48 h	72 h
0	100.01 ± 1.09	100.11 ± 0.76	100.02 ± 0.77
50	99.81 ± 1.98	98.93 2.19	88.84 ± 2.23 *
100	101.11 ± 1.18	97.24 ± 3.03	79.95 ± 2.96 **
150	99.54 ± 1.55	86.63 ± 2.83 *	60.03 ± 0.87 **
200	77.81 ± 2.99 **	65.83 ± 3.06 **	44.79 ± 1.97 **
300	63.05 ± 0.71 **	54.88 ± 1.44 **	30.71 ± 2.22 **

**$P < 0.01$ vs 0 μM *$P < 0.05$ vs 0 μM

2. Investigations and results

2.1. The effect of $CoCl_2$-induced hypoxia on cell viability

In order to verify the effect of $CoCl_2$ chemically induced hypoxia on the viability of HUVEC cells, the CCK8 method was used to detect the effects of different concentrations of $CoCl_2$ on HUVEC at different time points, and the cytotoxicity of $CoCl_2$ was evaluated. The results showed that $CoCl_2$ has little effect on cell viability at lower concentrations. According to the experimental purposes, the concentration of $CoCl_2$ was determined at 100 µM, the intervention time was 48 h, and the hypoxia induction model of HUVEC was constructed to observe the effect of drugs on cell viability. See both Table and Fig. 1.

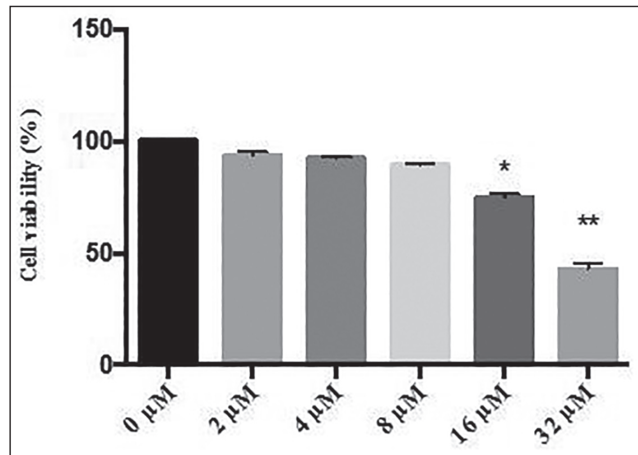

Fig. 1: Effects of drugs on hypoxic cell viability. **P < 0.01 vs 0 µM *P < 0.05 vs 0 µM.

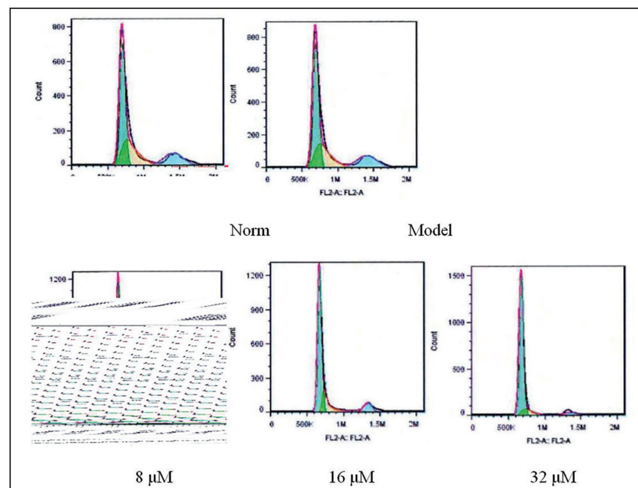

Fig. 2: Effect of drugs on the cell cycle.

2.2. Effects on the cell cycle

The results showed that, compared with the norm group, the model group exhibited no significant effect on cell cycle and no obvious cell proliferation inhibition. Compared with the model group, drug administered in different concentrations could affect the cell cycle, and cells in the G0/G1 phase were significantly increased, while the proportion of cells in S phase and G2/M phase were reduced. Under low concentration conditions, the cell cycle can be arrested in G0/G1 phase. As the drug concentration increased, the number of cells blocked in the G0/G1 phase increased, as shown in Fig. 2.

2.3. Effects on cell apoptosis

The results showed that there was no significant difference of the total apoptosis rate between the model and the norm group. The model group showed no significant effect on cell apoptosis.

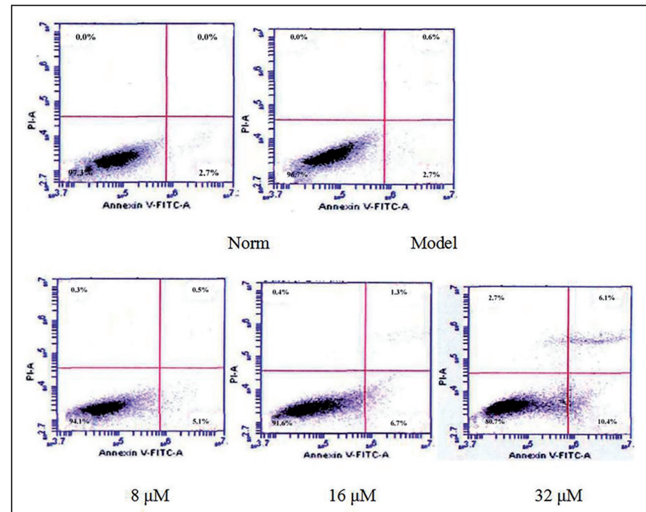

Fig. 3: Effect of drugs on apoptosis.

Different concentrations of drugs could induce apoptosis of hypoxia-induced HUVEC in a concentration-dependent way. Compared with the model group, apoptotic cells increased in the drug-administered groups, see Fig. 3.

2.4. Effect on cell lumen formation

After intervention by different concentrations of drugs, the number of complete lumens of HUVEC cells were significantly reduced, especially in the low concentration group. After addition of 16 µM drug, the lumen structure was destroyed, showing an incomplete and sparse network structure. However, in the high concentration group, cell agglutination exhibited, only one or none single lumen formed, contrast to the hypoxia group, see Fig. 4.

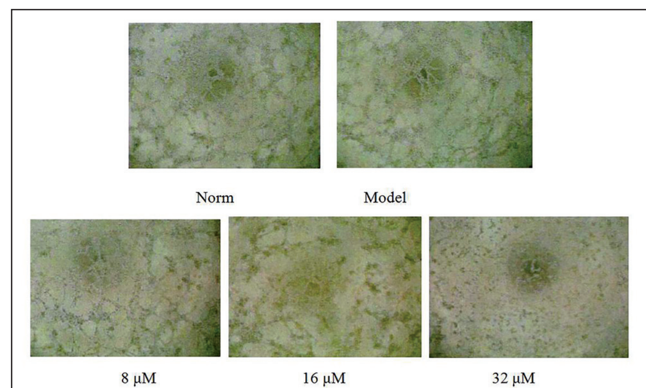

Fig. 4: Effect of drugs on cell lumen formation (´40).

2.5. Western blot analysis of HIF-1, GRP78, VEGF, VEGFR, PI3K, AKT, ERK, and FAK protein expressions

Western Blot results showed that, compared with the norm group, the hypoxia marker HIF-1a in the HUVEC of the model group was significantly increased, and the signaling pathways closely related to angiogenesis were VEGFA/VEGFR2, GRP78, PI3K/MT, ERK, and FAK proteins. The differences in protein expressions were significant as shown in Fig. 5.

2.6. Effect of silencing GRP78 on the expressions of angiogenesis-related signaling proteins

In order to verify the effect of silencing GRP78 on the expression and activities of angiogenesis signaling pathway proteins, the expressions of HIF-1α, VEGFA/VEGFR2, AKT, ERK and FAK

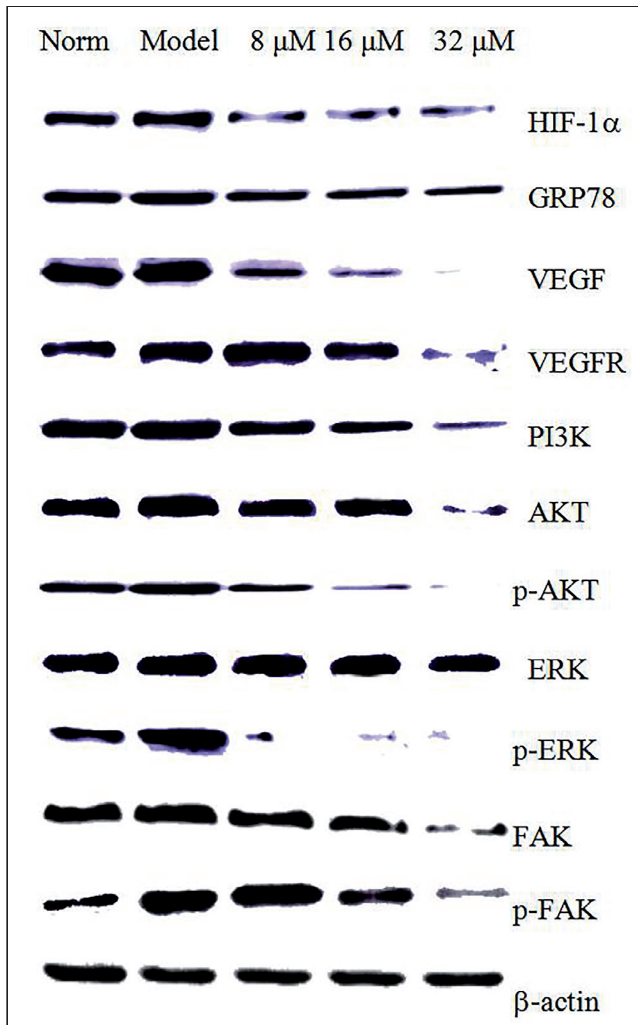

Fig. 5: Effect of drugs on various proteins.

proteins, and phosphorylated AKT, ERK and FAK levels were all detected by WB assay. It can be seen that the expressions of HIF-1α, VEGFA/VEGFR2, AKT, ERK and FAK proteins in siGRP78 group was significantly decreased, and the activities of phosphorylated AKT, ERK and FAK were significantly decreased, as shown in Fig. 6. The results suggested that GRP78 expression may affect angiogenesis-related signaling pathway protein expressions and AKT, ERK, FAK phosphorylation activities, thereby regulating tumor angiogenesis.

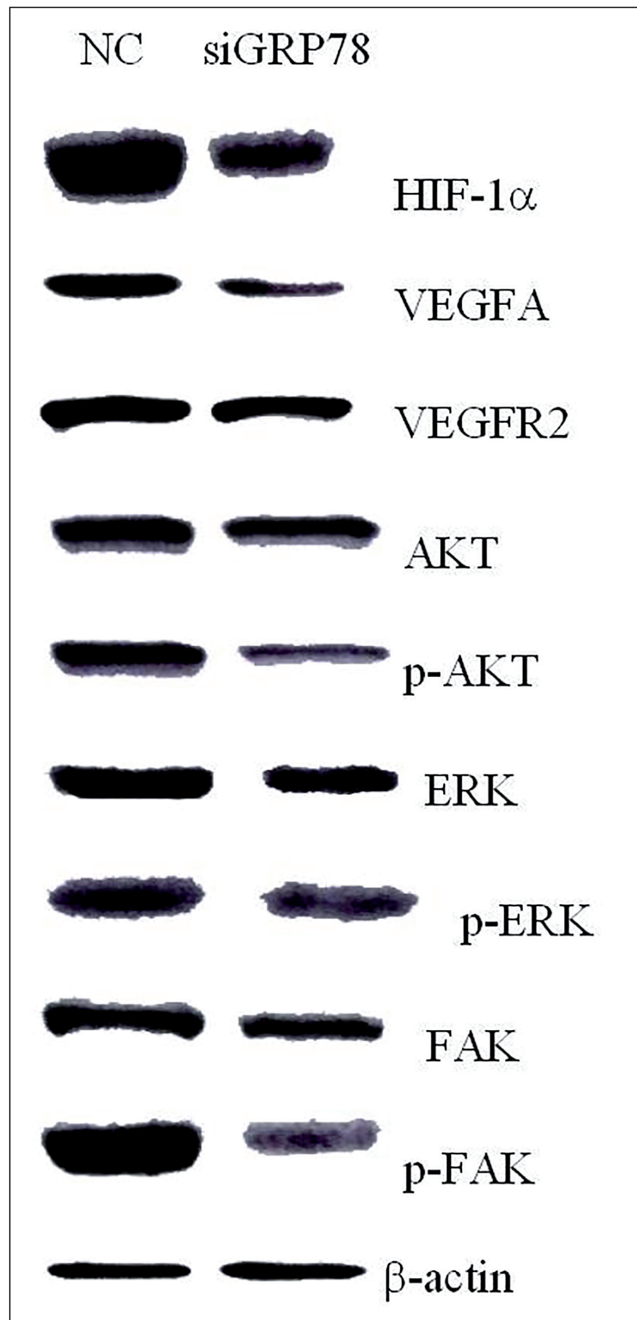

Fig. 6: Effect of silencing GRP78 on the expressions of angiogenesis-related signaling proteins.

3. Discussion

Current studies suggested that GRP78 can effectively regulate angiogenesis in tumor hypoxic microenvironment, it is therefore considered to be a new target for vascular access. GRP78 belongs to the heat shock protein 70 family and is an endoplasmic reticulum chaperone protein. It is a specific marker of endoplasmic reticulum stress. In the microenvironment of low glucose, hypoxia and acidosis, it can trigger unfolded protein reaction and induce endoplasmic reticulum. GRP78 expression would be elevated, and GRP78 in the cytoplasm is involved in multiple signaling pathways related with intracellular folding and apoptosis. GRP78 has been thought to be located in the lumen of the endoplasmic reticulum. As a transmembrane protein, it is located in the lumen of the endoplasmic reticulum with a carboxyl fragment. Recent studies have shown that GRP78 is not only found in the endoplasmic reticulum but also in the cell membrane, cytoplasm, mitochondria, nuclear and cell secretions. Highly expressed GRP78 can activate multiple signaling pathways and participate in tumorigenesis and development, which is closely related to tumor cell proliferation, anti-apoptosis, immune escape, and angiogenesis (Tsai et al. 2015; Miao et al. 2013; Luo and Lee 2013). Decreasing GRP78 expression in HUVEC significantly inhibits cell proliferation and is a key mediator of angiogenesis (Dong et al. 2011).

In the present study, the effect of silencing GRP78 on endothelial cell angiogenesis and related signaling pathways was observed by siRNA technique. Silencing GRP78 significantly reduce the ability of cells to form lumens. The result demonstrated that GRP78 expression is directly related to endothelial angiogenesis, and GRP78 signaling molecules are involved in angiogenesis. Silencing GRP78 reduced the expressions of HIF-la, VEGFA, VEGFR2, AKT, ERK, and FAK proteins and inhibited the phosphorylation of AKT, ERK and FAK. It was confirmed that GRP78 is involved in mediating the regulation of endothelial cell angiogenesis, and the mechanism is related to affecting HIF-la, VEGF/VEGFR, PI3K/AKT, MAPK and FAK. It is also proved that GRP78 may be the upstream signaling molecule of many important angiogenesis pathways. After silencing GRP78, the

activation and activities of downstream signaling pathways are inhibited. It is suggested that GRP78 is involved in mediating and regulating the important signaling pathways involved in angiogenesis.GRP78 has been shown to activate ERK, P13K/AKT, FAK and other signaling pathways. GRP78 can be increased by $CoCl_2$ and HIF-la stabilizers (Sawada et al. 2008), indicating that GRP78 may be a downstream target of HIF-1a under hypoxic conditions, working as an intermediate of HIF-1a involved in the regulation of energy metabolism in tumor cells. However, it is found that HIF-1a expression is decreased after silencing GRP78, suggesting that GRP78 may be silenced by SiRNA in the normoxic environment. As the angiogenesis signal pathway is complex and the cell growth conditions are different, the signal molecules are directly or indirectly regulated by multiple pathways, and there are also interactions, which needs further study.

In summary, the hypoxic microenvironment can enhance the angiogenesis of HUVEC, and the protein and gene expressions of multiple signaling pathways are elevated. Diosgenin can regulate the hypoxic microenvironment to inhibit HUVEC angiogenesis and reduce HUVEC activity, decrease angiogenesis pathway expression and consequently tumor growth and metastasis. GRP78 plays an important role in cell angiogenesis. The mechanism may be through the activation of GRP78 signaling pathways HIF-1a and VEGF/VEGF, as well as its downstream PI3K/AKT, MAPK, FAK pathways, or through directly activating PI3K/MT, MAPK and FAK which participated in tumor angiogenesis.

Diosgenin regulates the development of tumorigenesis by inhibiting the expression of tumor angiogenesis pathway through multiple whip points and pathways. However, the interrelationships between angiogenesis signaling pathways are synergistic. Due to various factors, anti-angiogenesis is a complex process. Therefore, it is proposed to use binding protein-specific inhibitors/activators in the future study. Further experimental research and mechanism discussion would provide more theoretical basis for the research of anti-tumor angiogenesis of diosgenin.

4. Experimental

4.1. Establishing the hypoxic cell model

Human umbilical vein endothelial cells (HUVEC) in logarithmic growth phase were re-suspended in RPMI-1640 complete medium (10 % FBS, 100 U/ml penicillin, and 100 g/ml streptomycin). The cells were inoculated into 96-well plates (100 ml/well) at the density of 5´10^4 cells/ml, and the cells were adhered to the incubator wall for overnight. The RPMI-1640 basal medium was starved for 24 h. The basal medium was discarded and replaced with RPMI-1640 complete medium. Different concentrations of $CoCl_2$ (0 mM, 50 mM, 10 mM, 150 mM, 200 mM and 300 mM) were added and intervened for 24 h, 48 h, and 72 h, respectively. A host of 10 ml of CCK8 solution was added to each well. The cells were incubated for 3 h in a humidified carbon dioxide incubator at 37 °C. The absorbance value at 450 nm was measured with a microplate reader, and the growth of the cells was observed.

4.2. CCK8 method for detecting cell viability

The established hypoxic cell model was used. The cells were inoculated in 96-well plates with 5´10^4/ml per well, and adhered overnight, then the serum was removed in serum-free medium for 24 h. According to the $CoCl_2$ kit, the final concentration is 100 mM for the hypoxic model. A total of 100 mM diosgenin in different concentrations (0 mM, 2 mM, 4 mM, 8 mM, 16 mM, and 32 M) was added for 48 h interaction. CCK8 solution at 10 ml/well was added to the culture plate which was then put in the incubator for 3 h. The absorbance value at 450 nm was determined by a microplate reader, and the effect of the concentration of diosgenin on the viability of HUVEC was observed.

4.3. Flow cytometry and Annexin V-FITC/PI staining to detect cell cycle and apoptosis

4.3.1. Flow cytometry for detecting cell cycle

The cells were seeded in a 6-well plate, and the control (norm) group was set up. The hypoxia (model) groups received different concentrations (8 mM, 16 mM, and 32 mM) of drug. The cells were cultured for 24 h. The cells were washed with PBS, digested with 0.25 % trypsin, then the cell suspension was collected and centrifuged at 800 rpm for 5 min. The supernatant was discarded, and the cells were re-suspended in PBS. After centrifugation, the supernatant was discarded, and the cells were re-suspended in PBS to make single-cell suspension, and the cell density was adjusted to 2×10^6/ml. The cells were quickly added to 3 times volume of 95% ethanol which was pre-cooled at 4 °C. The cells were agitated and fixed at 4 °C for more than 24 h, then sent for inspection. The cell proliferation index PI was calculated.

4.3.2. Annexin V-FITC/PI double labeling method for detecting apoptosis

HUVEC were obtained in the logarithmical growing phase. The cells were re-suspended evenly, and the cell concentration was adjusted. The cells were seeded in a 6-well plate at the density of 5´10^4/ml per well, and cultured overnight in a cell culture chamber with 5 % CO_2 at 37 °C. The medium was starved for 24 h. The cells were digested with trypsin without EDTA after 48 h according to the aforementioned grouping treatment. After collecting, centrifuging, re-suspending and counting, the cell concentration was adjusted to 1´10^6/ml, and 1 ml of the cell suspension was obtained to be centrifuged at 1000 rpm for 5 min. The supernatant was discarded, the cells were washed twice with PBS (pre-cooled at 4 °C), then centrifuged at l00 rpm for 5 min. The supernatant was discarded, the cells were collected, and 500 µl of binding buffer was added to suspend cells. A host of 5 µl of Annexin V-FITC was added and mixed gently. Another 5 µl of PI were added and then mixed softly. The cells were incubated at room temperature for 15 min in the darkness. Flow cytometry was performed to calculate the apoptosis rate of each group.

4.4. Tube formation observation of lumen formation

After routine culture, HUVEC was inoculated into 6-well plates at 5´10^4/ml/well, and cultured for 48 h according to the experimental method described above. The cells were digested, centrifuged, and re-suspended in serum-free RPMI-1640 medium. The cell numbers were counted. Cell concentration was adjusted to 2.5´l0^6/ml. The 96-well plate was placed in a 37 °C incubator for 60 min, then the plate was taken out and the Matrigel would be solidified at the bottom of the plate. A total of 100 mL cell suspension was inoculated on the Matrigel, with 3 parallel wells in each group, and then placed in an incubator for 6 h. Microscopically, five fields of view (X40) were taken and the endothelial cell lumen formation ability was evaluated by the number of lumen formation.

4.5. Western blot analysis of HIF-1, GRP78, VEGF/VEGFR, PI3K/AKT, ERK, and FAK protein expressions

Cells of each group were collected and then washed twice in PBS. A total of 400 µl of cell lysate was added to each flask, then 40 µl of 10 mmol/L PMSF was added. The culture flasks were gently shaken, then placed on ice for 10 min to make the cells lysate evenly. The cells were aspirated repeatedly with a sterile syringe. The lysed product was added to the EP tube, which was ice-bathed for 30 min, then centrifuged at 12000 x g for 15 min. The supernatant was transferred to a new EP tube, and the protein concentration was quantified by protein standard BC method.

Following this, each tube was added with 20 µl protein and 6XBuffer of every 100 µl and mixed. The tubes were boiled for 5 min, and then stored at -80 °C. The above samples were obtained, and the protein was separated by 12 % SDS-PAGE electrophoresis, and the separated protein bands were transferred to the PVDF membrane by wet method. The cells were closed at room temperature for 1 h, and then incubated overnight at 4 °C with the primary antibody (HIF-1a, GRP78, VEGF/VEGFR, PI3K/AKT, ERK, and FAK, all concentration at 1:1000), and then were washed three times with PBST. After incubation with a secondary antibody (1:1000) for 1 h, the cells were washed again with PBST three times. The cells were processed by chemiluminescence for color development and fixation. The expressions of each of the above proteins were determined.

4.6. Cell siRNAGRP78 transfection construction

The GRP78 siRNA sequence was designed and synthesized by Shanghai Jima Pharmaceutical Technology Co., Ltd. (Synthesis of 1 negative control sequence and 3 GRP78 sequences) as follows:

The cells were digested and resuspended in the complete medium. Following this, the cells were seeded into a 12-well plate at the density of 1´10^5 cells/ml, and placed in a 37 °C cell culture incubator. After 24 h of inoculation, the cell density reached 60-70 %. The OPTI was taken out from the 4 °C environment in advance to reach room temperature. The OPTI and siRNA were added in proportion to the RNase-free centrifuge tube, the siRNA was diluted to the desired concentration, mixed by pipetting, and put under room temperature for 5 min. The OPTI and Lipofectamine RNAiMAX transfection reagents were prepared by pipetting and mixing in an RNase-free tube. An equal volume of siRNA and IMAX transfection reagent were mixed by pipetting, and put at room temperature for 15 min. The culture supernatant was removed, and the cells were washed once with an appropriate amount of OPTP. The mixed transfection reagent was added to the culture well. After transfection for 6 h, the transfection reagent was removed and replaced with normal medium. The cells were collected after 48 h.

4.7. Western blot verification of siRNA transfection system

Cells were treated as in section 4.6., according to the Western blot procedures as described in the section 4.5.

4.8. Statistical processing

The experimental data were processed and analyzed by SPSS19.0 statistical software. Firstly, the normality test and the homogeneity test of variances were performed. Those who met the conditions were compared. The comparison between multiple groups of data was analyzed by one-way analysis of variance. With those who did not meet the conditions, data comparisons between two groups were made using the Wilcoxon rank sum test. Data results are expressed as mean±standard deviation. $P < 0.05$ was considered statistically significant.

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Conflict of interest: The authors declare that they have no competing interest.

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