



Inhibition of HIF-1a-mediated TLR4 activation decreases apoptosis and promotes angiogenesis of placental microvascular endothelial cells during severe pre-eclampsia pathogenesis



Lina Zhao^a, Ruixia Ma^a, Li Zhang^a, Xiaolan Yuan^a, Jinhua Wu^a, Lirong He^a, Guocheng Liu^{a,*}, Rui Du^{b,**}

^a Department of Obstetrics, Guangdong Woman and Children Hospital, Guangzhou, Guangdong, China

^b Department of Pathology, Guangdong Woman and Children Hospital, Guangzhou, Guangdong, China

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ABSTRACT

Objective: Hypoxia-induced factor 1a (HIF-1a) and Toll-like receptor 4 (TLR4) are involved in pre-eclampsia (PE) pathogenesis. However, little is known about their relationships. This study aimed to investigate the interaction of HIF-1a and TLR4 in PE pathogenesis.

Methods: The expression of HIF-1a and TLR4 were analyzed by qRT-PCR. Cellular PE model was established by hypoxia/reoxygenation treatment of human placental microvascular endothelial cells (hPMEC). Cell proliferation, apoptosis, invasion and migration were analyzed by CCK-8, flow cytometry, Transwell and scratch adhesion test, respectively. Angiogenesis was performed by tube formation, Ang-1 in culture supernatant was analyzed by ELISA.

Results: HIF-1a and TLR4 expression were significantly elevated in placental tissues from early-onset and late-onset severe pre-eclampsia patients compared with control, with increased Bax, TRIF and PUMA, and decreased Bcl-2 and VEGFA; Down-regulation of HIF-1a expression decreased TLR4 expression, promoted proliferation, invasion, migration and angiogenesis but suppressed apoptosis in cellular model. In addition, silencing HIF-1a and TAK232 treatment synergistically promoted some more proliferation, invasion, migration and angiogenesis but suppressed apoptosis in cellular model.

Conclusion: HIF-1a could promote hPMEC apoptosis by regulating TLR4 expression during PE pathogenesis.

1. Introduction

Pre-eclampsia (PE) is a common pregnancy-associated disorder featured by hypertension with high systolic blood pressure and proteinuria, or abnormally high serum creatinine, as well as increased liver transaminase and damaged liver function, thrombocytopenia, cerebral problem and pulmonary oedema [1]. Globally, the prevalence of pre-eclampsia was reported to over 5%, which has been responsible for more than 50 000 deaths each year and listed as one of the major causes of maternal mortality [1,2]. Moreover, PE has also been associated with increased risk of cardiovascular complications and diabetes mellitus. The pathogenesis of PE was linked with multiple genetic and environmental factors [1,3]. According to the time of onset, PE could be

categorized into two major subtypes, including the early-onset PE which occurs before 33 gestational weeks and the late-onset PE at or after 34 weeks' gestation [2,4]. Although late-onset PE accounts for more than 80% PE incidences, the high maternal mortality rates of PE patients were mainly attributed to the early onset subtype [1]. However, in clinics, few effective treatments are currently available for PE patients except the delivery of fetus and placenta [1,2].

In pathogenesis of PE, the defects in spiral artery remodeling could lead to placental ischemia and the hypoxia-reperfusion (H/R) damages of trophoblasts, which then cause the increase of syncytiotrophoblast microparticles, dysregulation of cytokine expression, and the imbalance of angiogenic and anti-angiogenic factors in maternal circulation, finally inducing dysfunction of placental microvascular endothelial cells

* Corresponding author. Department of Obstetrics, Guangdong woman and Children Hospital, No. 521, Xingnan Avenue, Panyu District, Guangzhou City, 510000, Guangdong, China.

** Corresponding author. Department of Pathology, Guangdong woman and Children Hospital, No. 521, Xingnan Avenue, Panyu District, Guangzhou City, 510000, Guangdong, China.

E-mail addresses: hnlguocheng@126.com, liuguocheng168@yeah.net (G. Liu), Xzf192@sohu.com (R. Du).

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and PE onset [1]. During development of early-onset PE, low oxygen tension and interruption of oxygen-sensing function of placentas could induce the greatly enhanced expression of hypoxia inducible factor-1 (HIF-1 α) in placental tissues [5,6]. The sustained high-expression of HIF-1 α in placental tissues and maternal circulation contributed to the shallow trophoblast invasion of the spiral arteries during PE pathogenesis [5,7]. The hypoxia and oxidative stresses in placental tissues in PE patients could also cause the abnormal expression of TGF- β (tumor growth factor beta) and other functional genes [8,9]. For instance, the expression and variation of vascular endothelial growth factors (VEGF), have been frequently detected in patients with PE [10–12]. Moreover, the matrix metalloproteinase (MMP) were also shown to be associated with pre-eclampsia [13]. However, the pathogenesis and pathophysiology of PE, especially the downstream molecular events of HIF-1 α expression, still remains incompletely understood.

Toll-like receptors (TLRs), as integral membrane glycoproteins featured by leucine-rich motifs, are widely expressed in epithelial cells, as well as a placental immune cells and chorion trophoblast placental immune cells, act as innate immune cells receptors and regulate immune responses [14,15]. Among ten members of the TLR family, TLR4 (Toll-like receptor 4) could be stimulated by the lipopolysaccharide (LPS) of gram-negative bacterium, and also respond to signaling molecules released by damaged cells during PE pathogenesis such as fibrinogen and heat-shock proteins [16]. TLR4, together with MyD88 (myeloid differentiation primary response 88) and NF- κ B (Nuclear factor kappa B), was reported to be increasingly expressed in trophoblasts and umbilical cord blood mononuclear cells in women with PE [16]. In addition, high TLR4 expression was also detected in various other cell types in placenta, including amniotic cells, intermediate and extravillous trophoblast during normal pregnancy and pathogenesis of PE [14]. Activation of the TLR4 signaling induces NF- κ B activation, cytokine synthesis, TLR-triggered inflammatory responses and placental cytotrophoblast apoptosis [16]. However, little is known about the mechanisms under TLR4 expression alteration in PE pathogenesis.

In this study, the expression of HIF-1 α and TLR4 in placental tissues of women with early-onset and late-onset severe pre-eclampsia were investigated, followed by further analysis using a cellular PE model induced by of hypoxia and reperfusion, which might provide novel insights into the pathogenic roles of HIF-1 α and TLR4 during PE onset.

2. Material and methods

2.1. Clinical tissue collection

The human placental villus tissues were collected from 30 women with early-onset severe pre-eclampsia and 30 women with late-onset severe pre-eclampsia, 60 women with normal pregnancy, who were admitted into the Hospital of Guangdong woman and Children Hospital between 2017 and 2018 (Table 1). The placental tissues were immediately frozen in liquid nitrogen for total RNA or protein extraction. The experimental procedures were approved by the Ethics Committee

Table 1
Clinical data of all the patients.

| women Characteristic | E-NP (n = 30) | L-NP (n = 30) | EOPE (n = 30) | LOPE (n = 30) |
|----------------------|--|--|---|---|
| Ages(Years) | 30.6 \pm 5.4 | 30.1 \pm 4.4 | 33.1 \pm 5.2 | 31.6 \pm 4.7 |
| Weight(kg) | 59.9 \pm 8.9 | 63.7 \pm 3.7 | 61.1 \pm 10.6 | 69.8 \pm 12.6 |
| Gestational weeks | 25.4 \pm 5.2 | 37.5 \pm 1.7 | 26.17 \pm 5.4 | 37.7 \pm 1.8 |
| Blood pressure | 109.3 \pm 12.4 ^a /62.4 \pm 5.9 ^b | 110.6 \pm 11.2 ^a /72 \pm 5.7 ^b | 137.7 \pm 31.6 ^a /84.4 \pm 14.7 ^b | 131.6 \pm 26.1 ^a /83.1 \pm 13.5 ^b |
| medical history | No special | No special | No special | No special |
| Delivery times | 2.7 \pm 0.3 ^c /0.3 \pm 0.8 ^d | 2.3 \pm 0.8 ^c /0.8 \pm 0.5 ^d | 2.4 \pm 1.0 ^c /0.4 \pm 0.6 ^d | 2.9 \pm 1.6 ^c /0.8 \pm 0.7 ^d |
| delivery outcome | 12 ^e /3 ^f /3 ^g /12 ^h | 17 ^e /13 ^g | 13 ^e /9 ^f /4 ^g /4 ^h | 9 ^f /21 ^g |
| Urine protein | 0 ⁱ /30 ^j | 0 ⁱ /30 ^j | 14 ⁱ /16 ^j | 13 ⁱ /17 ^j |

Note: a, systolic pressure; b, diastolic pressure; c, number of pregnancy; d, outcome of pregnancy; e, induced labor; f, cesarean section; g, eutocia; h, continued pregnancy; i, Number of PE women with urine protein; j, Number of PE women with negative urine protein.

of the Guangdong woman and Children Hospital [NO. 201801028], and written consents were obtained from each subject in advance.

2.2. Cell culture, treatment and transfection

Human placental microvascular endothelial cells (hPMEC) isolated from human placenta were purchased from the ScienCell Research Laboratories (#7100) and cultured in Endothelial Cell Medium (ECM, Cat. #1001) at 37 °C in a humidified atmosphere with supply of 5% CO₂. Silencing of HIF-1 α expression in hPMEC was done by transfection with siRNAs (GenePharma, Shanghai) specifically targeting HIF-1 α using the Lipofectamine™ 2000 Transfection Reagent (Thermo Fisher Scientific). For inhibition of TLR4 expression, the hPMEC cells were treated with 5 μ M TAK242 (Sigma; #614316) for 2 h after cell transfection, followed by the hypoxia and reoxygenation treatment.

2.3. Cellular hypoxia and reoxygenation model

An *in vitro* cellular model of early-onset pre-eclampsia was established by treating cultured hPMEC with hypoxia followed by reoxygenation. The hypoxia and reoxygenation treatment were carried out following cell transfection as previously described [17]. Briefly, after being cultured under normal conditions overnight, hPMEC cells were rinsed with fresh culture medium for two times and cultured in Tri-Gas CO₂ Incubators (Thermo Fisher Scientific) with a supply of 2% oxygen for constant 4 h, followed by culture under normal conditions (reoxygenation).

2.4. Quantitative RT-PCR

Total RNA samples were extracted from placental tissues using the Trizol kit (Takara, 9109), followed by rinsing with 75% alcohol. The synthesis of cDNA was finished using the Bestar™ qPCR RT kit (DBI; #2220) from 2 μ g RNA samples. The relative mRNA levels were determined by real-time quantitative PCR method using the Bestar™ qPCR MasterMix (DBI; #2043). The PCR process was done on an ABI Real time PCR machine using the following settings: 95 °C for 2 min, followed by 40 cycle of 94 °C for 20 S, 58 °C for 20 S and 72 °C for 20 S. Data from more than three biological replicates were subjected to final quantitation and statistical analysis using the 2^{- $\Delta\Delta$ Ct} method. GAPDH was served as the internal reference. The sequences of primers used for quantitative RT-PCR analysis are listed in Table 2.

2.5. Western blotting

Total protein samples from placental tissues and cultured hPMEC cells were extracted using Cell Lysis buffer (#P0013; Beyotime, China). Protein abundances were determined using the Pierce™ BCA Protein Assay Kit (#23227; Thermo Fisher Scientific). Approximately 30 μ g proteins were loaded in 10–12% SDS-PAGE electrophoresis and transferred onto PVDF membranes (Millipore). The membranes were then

Table 2
Primers used in the study.

| Gene | Sequence (5'- 3') | Product Length (bp) |
|----------|---------------------------|---------------------|
| GAPDH.F | TGTTTCGTCATGGGTGTGAAC | 154 |
| GAPDH.R | ATGGCATGGACTGTGGTCAT | |
| HIF-1a.F | GAACGTCGAAAAGAAAAGTCTCG | 124 |
| HIF-1a.R | CCTTATCAAGATGCGAACTCACACA | |
| TLR4.F | AGACCTGTCCCTGAACCTAT | 147 |
| TLR4.R | CGATGGACTTCTAAACCAGCCA | |

blocked with 5% lipid-free milk and incubated with the following primary antibodies overnight at 4 °C: *anti*-Bax (Bcl2-associated X protein) (1: 1000, ab32503, Abcam), *anti*-Bcl-2 (B Cell Lymphoma 2) (1: 2000, ab32124, Abcam), *anti*-HIF-1 α (1: 400, ab51608, Abcam), *anti*-TLR4 (1: 800, ab13556, Abcam), *anti*-TRIF (Toll/IL-1 receptor domain-containing adaptor inducing IFN- β) (1: 1000, ab13810, Abcam), anti-PUMA (P53 upregulated modulator of apoptosis) (1: 1000, ab33906, Abcam), *anti*-VEGFA (1: 1500, ab52917, Abcam) and *anti*-GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) (1: 10000, ab8245, Abcam). After being incubation with secondary antibodies for 1 h, the immuno-complexes were detected using the WBKLS0500 Immobilon Western Chemilum HRP substrate (Millipore) and analyzed using the Image-Pro Plus 6.0 software.

2.6. CCK-8 assay

The proliferation rates of cultured hPMEC cells after specified transfection and treatments were done using the Cell Counting 8 kit (#C0038; Beyotime). Briefly, hPMEC cells in the logarithmic growth phase were seeded in the 96-well plates, incubated with CCK-8 solution at 37 °C for 24 h in a humidified atmosphere with supply of 5% CO₂. Proliferation rates were finally evaluated by measuring the absorbances at 450 nm using microplate reader. OD450 values from at least three biological replicates were collected for quantitation and statistical analysis.

2.7. Flow cytometry

The apoptosis of hPMEC cells were analyzed by flow cytometry using the Annexin V-FITC Apoptosis Staining Detection Kit (#ab14085; Abcam). In brief, cultured hPMEC cells were subjected to staining with 5 μ l Annexin V-FITC and 5 μ l Propidium Iodide for 5–10 min at dark, and finally analyzed using a flow cytometer. For statistical significance analysis, the flow cytometry assay was biologically repeated for at least three times.

2.8. Cell migration and invasion analysis

The migration capacities of hPMEC cells were analyzed by wound healing assay. In brief, cells seeded in 24-well plates (approximately 3.0×10^5 cells per well) and cultured under normal conditions for 24–48 h until the formation of cell monolayer. A straight scratch was then made in the middle of cell plate well using a sterile pipette tip, and the wound healing was evaluated using a microscope after 48 h. Cell invasion abilities in this study were evaluated using the Transwell system (Corning, USA) which was pre-coated with Matrigel (BD Biosciences). HPMEC cells were first seeded in the upper chambers of the Transwell plates filled with serum-free culture medium, and after culture under normal conditions for 48 h, the cells in the lower surface of the Matrigel membranes were stained with 0.1% crystal violet for cell counting and observation using light microscope.

2.9. Tube formation assay

The *in vitro* angiogenesis of hPMEC cells were assessed by the tube

formation assay. Briefly, 200 μ l reduced growth factor matrigel (#354234; BD) was added into each well of a 24-well plate, which was incubated at 37 °C for 1 h with supply of 5% CO₂ for solidification. Cultured cells were collected and resuspended in ECM, seeded into plate wells (1 $\times 10^5$ cells per well) and cultured under normal conditions for 48 h with close observation. The formation of tubes was photographed and at least three biological replicates were done for evaluation of cell angiogenesis ability.

2.10. Enzyme linked immunosorbent assay

The enzyme linked immunosorbent assay (ELISA) was performed to detect the supernatant of hPMEC cell cultures using the Human Angiotensin-1 (ANG-1) ELISA Kit (Nova Lifesciences). In brief, cell supernatants were incubated with Detection A solution at 37 °C for 1 h, washed for three times, incubated with Detection Reagent B working solution at 37 °C for 45 min, mixed with 90 μ l Substrate Solution followed by incubation at 37 °C for 15 min. Ang-1 levels were finally determined by measuring the absorbances at 450 nm using a plate reader.

2.11. Statistics

Quantitative data from more than three biological repeats were presented as mean \pm SD and analyzed using the SPSS 18.0 software. The significance of differences between groups were assessed by the Student T-test (among two groups) and analysis of variance (ANOVA) test (among more than three groups). Significant differences were finally defined by a *P* value of < 0.05.

3. Results

3.1. Increased HIF-1 α and TLR4 expression in PE tissues

To assess roles of HIF-1 α and TLR4 in PE progression, the placental tissues were collected from early-onset and late on-set PE severe patients, as well as women with normal pregnancy. Clinical data of all the patients are listed in Table 1. As Fig. 1A and B shown, compared with women with normal pregnancy less than 34 weeks (E-NP) and women with normal pregnancy more than 34 weeks (L-NP) groups, the mRNA expression of HIF-1 α and TLR4 was significantly elevated in early-onset severe PE patients less than 34 weeks (EOPE) and late-onset severe PE patients more than 34 weeks (LOPE) groups. And the mRNA expression of HIF-1 α and TLR4 was also found significantly elevated in placental villus tissues from E-NP group than that in placental villus tissues from L-NP group. In addition, the protein abundances of HIF-1 α , TLR4, TRIF, Bax and PUMA were significantly increased in EOPE and LOPE groups compared with E-NP and L-NP groups, while Bcl-2 and VEGFA showed the opposite trend. (Fig. 1C and D). Further analysis showed that there were no changes in the expression of Bcl-2 and VEGFA at protein level between LOPE and L-NP group.

3.2. Silencing HIF-1 α promotes cell proliferation, invasion, migration and represses apoptosis in PE model

To investigate the pathogenic functions of HIF-1 α in PE development, the hPMEC cells were transfected with siRNAs to suppress HIF-1 α expression, which were then subjected to the hypoxia and reoxygenation treatment for *in vitro* cellular PE model establishment. Through CCK-8 assay, we found that the hypoxia and reoxygenation caused significantly decrease of the proliferation capability of hPMEC compared with cells under normal conditions while silence of HIF-1 α expression by siRNA transfection restored the proliferation rates of hPMEC cells compared with cells transfected with NC siRNAs at 48 h and 72 h (Fig. 2A). Also, the percentages of apoptotic hPMEC cells was remarkably increased in cells treated with hypoxia and reoxygenation, which was significantly repressed by HIF-1 α siRNA transfection

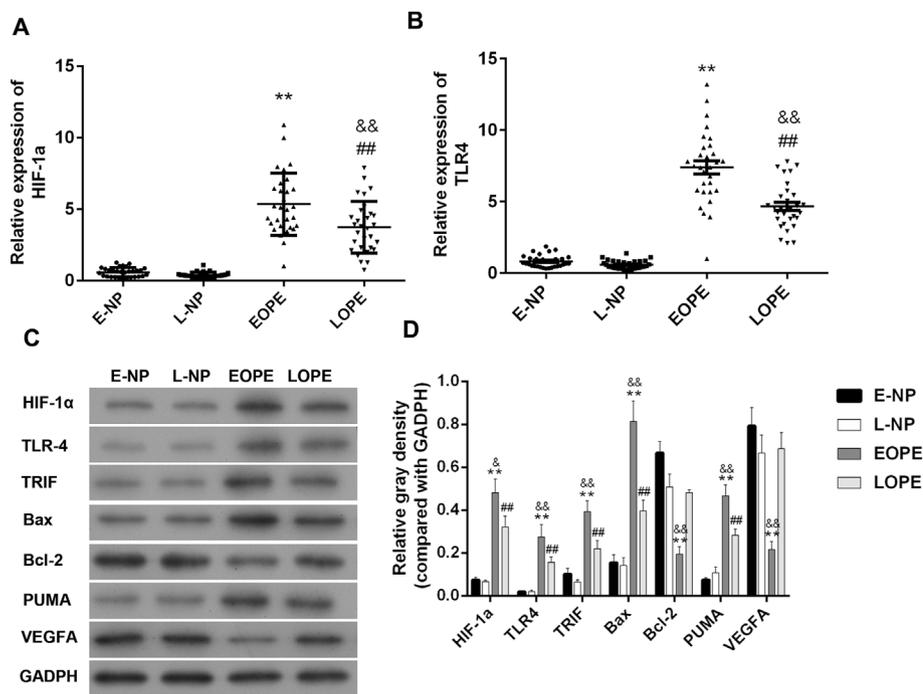


Fig. 1. Elevated HIF-1α and TLR4 expression in placental tissues of PE patients.

Relative mRNA expression of HIF-1α (A) and TLR4 (B) in E-NP, L-NP, EOPE and LOPE groups, respectively. (C) The protein abundances and relative gray density (D) of HIF-1α, TLR4, Bax, Bcl-2, TRIF, PUMA and VEGFA in E-NP, L-NP, EOPE and LOPE groups, respectively; GAPDH was used as the internal standard. E-NP, L-NP, EOPE and LOPE groups represented women with normal pregnancy (less than 34 weeks), women with normal pregnancy (more than 34 weeks), placental tissues from early-onset severe PE patients (less than 34 weeks) and late-onset severe PE patients (more than 34 weeks), respectively. PE: pre-eclampsia; Bax: Bcl2-associated X protein; Bcl-2: B Cell Lymphoma 2; HIF-1α: Hypoxia-inducible factor 1α; TLR4: Toll-like receptor 4; TRIF: Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta; PUMA: P53 up-regulated modulator of apoptosis; VEGFA: Vascular endothelial growth factor A; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. **, ##, and && represented the significance between EOPE and E-NP, LOPE and L-NP, EOPE and LOPE, respectively. **, ##, and && , $P < 0.01$.

compared with cells transfected with NC siRNAs (Fig. 2B and C). Using the Transwell system, it showed that the invasion capability of hPMEC cells was markedly impaired by the hypoxia and reoxygenation treatment while silencing HIF-1α recovered its invasive potential to a considerable degree (Fig. 2D and F). In addition, the wound healing assay demonstrated that hypoxia and reoxygenation significantly decreased the migration capability of hPMEC cells, but transfection with HIF-1α siRNAs significantly promoted the migration of hPMEC cells undergoing hypoxia and reoxygenation treatment in comparison with cells transfected with NC siRNAs (Fig. 2E and G). These results showed that HIF-1α plays critical roles during PE pathogenesis by regulating the proliferation, apoptosis, invasion and migration of hPMECs.

3.3. Silencing HIF-1α promotes angiogenesis and changes the apoptosis related protein expression in cellular PE model

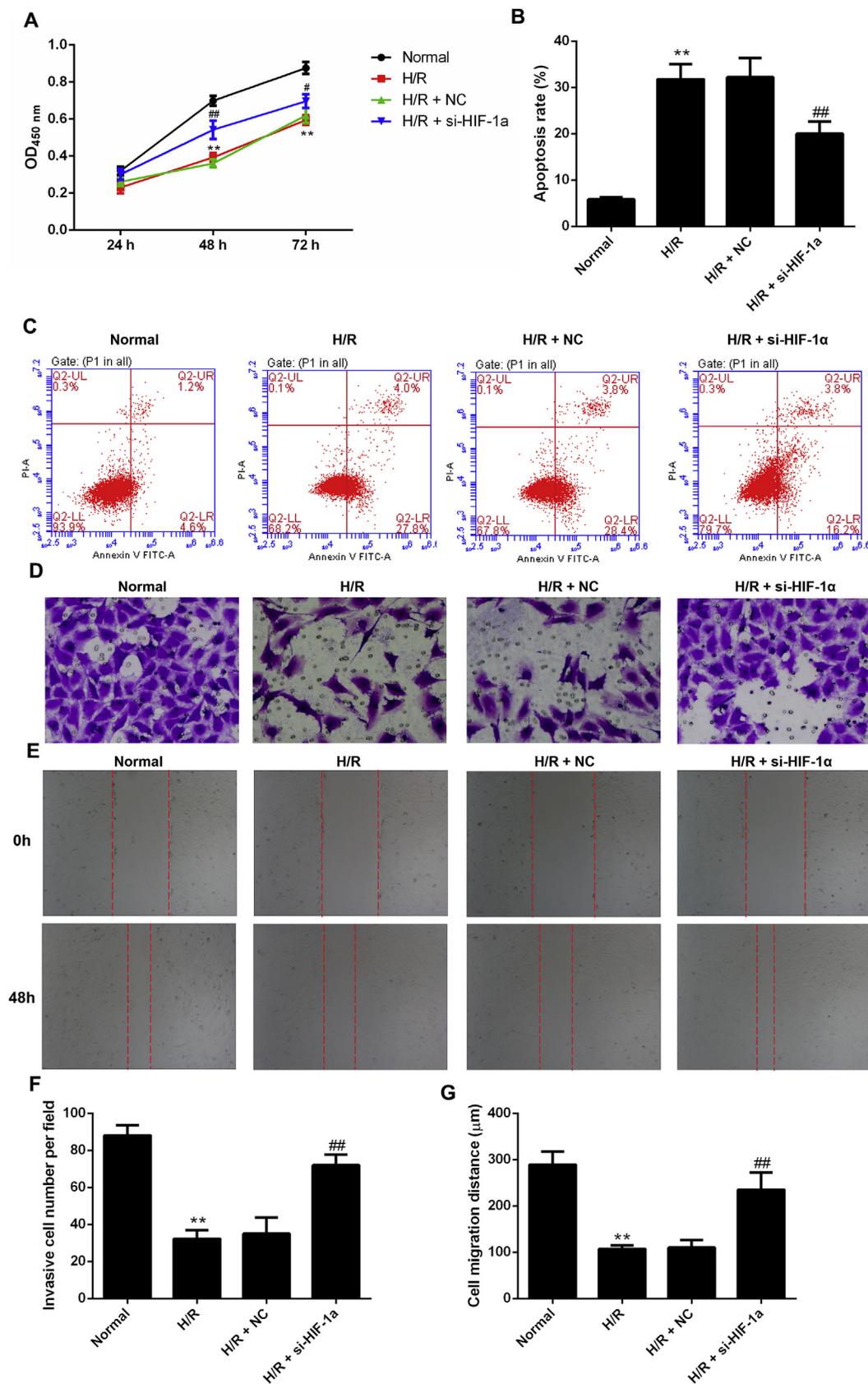
For more understanding of cellular processes regulated by HIF-1α, the expression of genes associated with angiogenesis and apoptosis were further investigated in supernatant from cellular PE model. Firstly, we found that the angiogenic ability of hPMEC cells was greatly suppressed after hypoxia and reoxygenation than that under normal condition while significantly enhanced in silencing HIF-1α group compared with H/R with NC group by tube formation assay (Fig. 3A and B). We also found that the concentration of ANG-1 in supernatant of hPMEC cell culture after hypoxia and reoxygenation treatment was significantly decreased compared with cells under normal culture conditions while silencing of HIF-1α gene expression recovered the ANG-1 level to a certain extent (Fig. 3C). Consistent with our above analysis in placental tissues, we found that the abundances of HIF-1α, TLR4, Bax, TRIF and PUMA in hPMEC cells after hypoxia and reoxygenation treatment were remarkably elevated in comparison with cells under normal condition. However, Bcl-2 and VEGFA protein abundances in cells after hypoxia and reoxygenation treatment were significantly decreased compared with normal control (Fig. 3D and E) while the expressional changes of these proteins induced by hypoxia and reoxygenation treatment were all inhibited by siRNA-mediated HIF-1α silencing in cellular PE model (Fig. 3D and E). These results demonstrated that HIF-1α played an important role in regulating angiogenesis and apoptosis during PE pathogenesis.

3.4. Synergistic action of HIF-1α and TLR4 on hPMEC cell function regulation

To further investigate the relationship between HIF-1α and TLR4 during PE progression, TLR4 inhibitor TAK242 were independently or co-transfected with HIF-1α siRNA to hPMEC cells. Firstly, compared with H/R group, the proliferation rates of hPMEC cells was significantly elevated by TAK242 treatment at 48 h and 72 h, and TAK242 co-transfected with HIF-1α siRNA group caused even greater increase of hPMEC cell proliferation rates than that in transfected with NC siRNA group (Fig. 4A). Furthermore, the apoptosis rates of hPMEC cells in H/R with TAK242 group was remarkably reduced than that in H/R group, and co-treatment with TAK242 and HIF-1α siRNAs almost recovered the apoptosis rates of hPMEC cell to a normal level (Fig. 4B and C). Moreover, the TAK242 could improve the invasion capability of hPMEC cells compared with H/R group and TAK242 co-transfected with and HIF-1α siRNAs was also significantly improved the invasion capability of hPMEC cells compared with co-transfected with NC group (Fig. 4D and F). In addition, the wound healing assay revealed similar alteration of hPMEC cell migration ability after combined treatments with TAK242 and HIF-1α siRNAs (Fig. 4E and G). These analyses proved the synergistic roles of HIF-1α and TLR4 exert in repressing proliferation, migration, invasion and promoting apoptosis of cellular PE model.

3.5. Angiogenesis and apoptosis pathways regulated by HIF-1α and TLR4 in cellular PE model

We further analyzed the effect of HIF-1α and TLR4 on angiogenesis. As shown in Fig. 5, TAK242 treatment dependently or combined with HIF-1α siRNAs remarkably recovered the angiogenic ability of hPMEC cells after hypoxia and reoxygenation (Fig. 5A and B). To provide more insights into the molecular events influenced by HIF-1α silencing and TLR4 inhibition, ANG-1 and several functional proteins involved in cell angiogenesis and apoptosis were also investigated in cellular PE model. Further analysis showed that the ANG-1 levels were significantly increased in supernatant of hPMEC cells with TAK242 treatment compared with H/R treatment, and the ANG-1 levels was also elevated in combined TAK242 and HIF-1α siRNAs group compared with TAK242 and NC group (Fig. 5C). Consistently, the levels of HIF-1α, TLR4, TRIF,



(caption on next page)

Bax and PUMA proteins in hPMEC cells undergoing hypoxia and reoxygenation were significantly decreased by TAK242 treatment. However, Bcl-2 and VEGFA protein levels in cells treated with TAK242 were

significantly elevated (Fig. 5D–F). And combined treatments with TAK242 and HIF-1α siRNAs caused even greater alterations of the abundances of these proteins in hPMEC cells after hypoxia and

Fig. 2. HIF-1 α silencing influences hPMEC proliferation, apoptosis, invasive and migration.

(A) Proliferation rates of hPMEC cells transfected with HIF-1 α siRNAs after hypoxia and reoxygenation. Cell proliferation rates were determined by the CCK-8 assay; (B) The apoptosis rates of hPMEC and cell apoptosis was evaluated by flow cytometry (C) transfected with HIF-1 α siRNAs after hypoxia and reoxygenation was quantified; (D) Invasion capacities of hPMEC cells and cell number per field (F) transfected with HIF-1 α siRNAs and treated with hypoxia and reoxygenation. The Transwell system was used to analyze cell invasion ability. (E) Migration capabilities of hPMEC cells and cell migration distance (G) with silenced HIF-1 α expression after hypoxia and reoxygenation treatment. Wound healing assay was done to measure cell migration ability. H/R: hypoxia and reoxygenation; HIF-1 α : Hypoxia-inducible factor 1 α ; NC: negative control; si-HIF-1 α : siRNAs targeting HIF-1 α ; OD450: absorbance at 450 nm ** and ## represented the significance between H/R and normal group, H/R with NC and H/R with si-HIF-1 α groups, respectively. ** and ##, P < 0.01.

reoxygenation treatment (Fig. 5D–F).

4. Discussion

The cell damages caused by hypoxia-reperfusion (H/R) in the placental tissues are key events during the pathogenic development of severe pre-eclampsia, but the molecular mechanism remains poorly understood [1,3]. The induced high expression of HIF-1 α and TLR4 genes were previously observed in placental tissues and cells associated with PE progression [5,14], however little is known their potential interaction during pathogenic development of severe PE. The human placental microvascular endothelial cells (hPMEC) act as major component of the placental vascular bed and are closely associated with placental angiogenesis and remodeling, placental angiokinesis and blood flow homeostasis in placenta [18]. The vascular defects in human placenta caused by endothelial cell dysfunction, such as human placental microvascular endothelial cells, were shown to closely associated with various human disorders including intrauterine growth restriction,

placental insufficiency and also and pre-eclampsia [19–21]. The association of hPMEC with pre-eclampsia development make it an ideal *in vitro* model for study of the cellular and molecular events associated with pre-eclampsia pathogenesis. Subsequently, the roles of interaction between HIF-1 α and TLR4 in H/R damages of hPMEC was investigated in the present study, aiming to provide novel mechanisms underlying PE pathogenesis.

The expressional alterations of HIF-1 α and TLR4 genes in the placental tissues of women with early-onset and late-onset severe pre-eclampsia, as well as those undergoing normal pregnancy, were first confirmed, and we found both genes were significantly elevated in severe pre-eclampsia tissues, especially those early-onset PE tissues. The high expression of HIF-1 α and TLR4 genes in placental tissues from PE patients indicated the potential pathogenic functions of them during PE pathogenesis, which were consistent with previous references [22–24]. For investigation of the roles of HIF-1 α and TLR4 in vascular endothelial cell damages during PE development, the hPMEC cells were treated with hypoxia and reoxygenation, which induced great

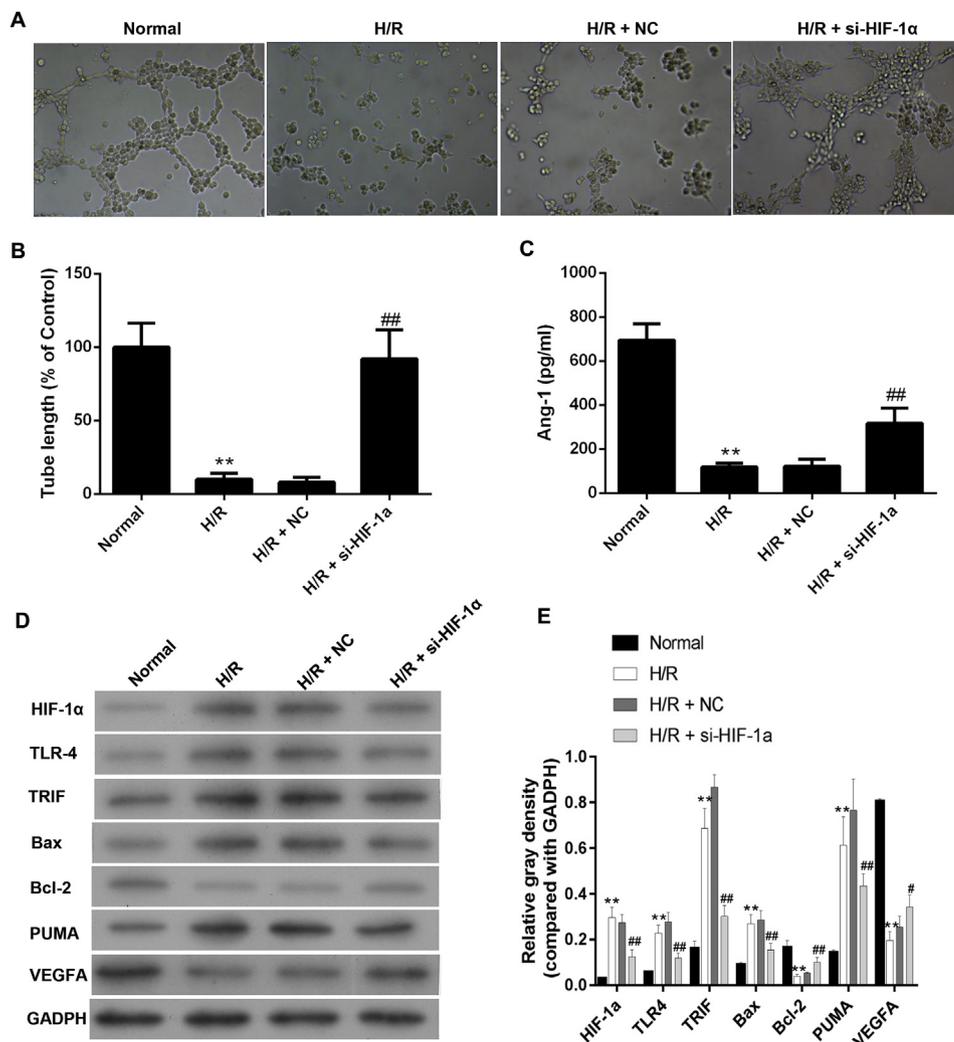


Fig. 3. Molecular pathways regulated by HIF-1 α in hPMEC cells after H/R treatment.

The angiogenic capability (A) and tube length (B) of hPMEC cells transfected with siRNAs targeting HIF-1 α and treated with hypoxia and reoxygenation. Angiogenesis in hPMEC cells was evaluated by tube formation assay. (C) Changes of ANG-1 levels in the supernatants of hPMEC cell culture induced by HIF-1 α siRNAs after hypoxia and reoxygenation treatment. ANG-1 levels in cell culture supernatants were analyzed by ELISA. (D) The protein abundances and relative gray density (E) of HIF-1 α , TLR4, TRIF, Bax, Bcl-2, PUMA and VEGFA in hPMEC cells transfected by HIF-1 α siRNAs after hypoxia and reoxygenation treatment. Western blotting was performed to determine protein levels in hPMEC cells. GAPDH was used as the internal standard. H/R: hypoxia and reoxygenation; NC: negative control; siHIF-1 α : siRNAs targeting HIF-1 α ; ANG-1: Angiopoietin-1; Bax: Bcl2-associated X protein; Bcl-2: B Cell Lymphoma 2; HIF-1 α : Hypoxia-inducible factor 1 α ; TLR4: Toll-like receptor 4; TRIF: Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta; PUMA: P53 upregulated modulator of apoptosis; VEGFA: Vascular endothelial growth factor A; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. ** and ## represented the significance between H/R and normal group, H/R with NC and H/R with si-HIF-1 α groups, respectively. ** and ##, P < 0.01.

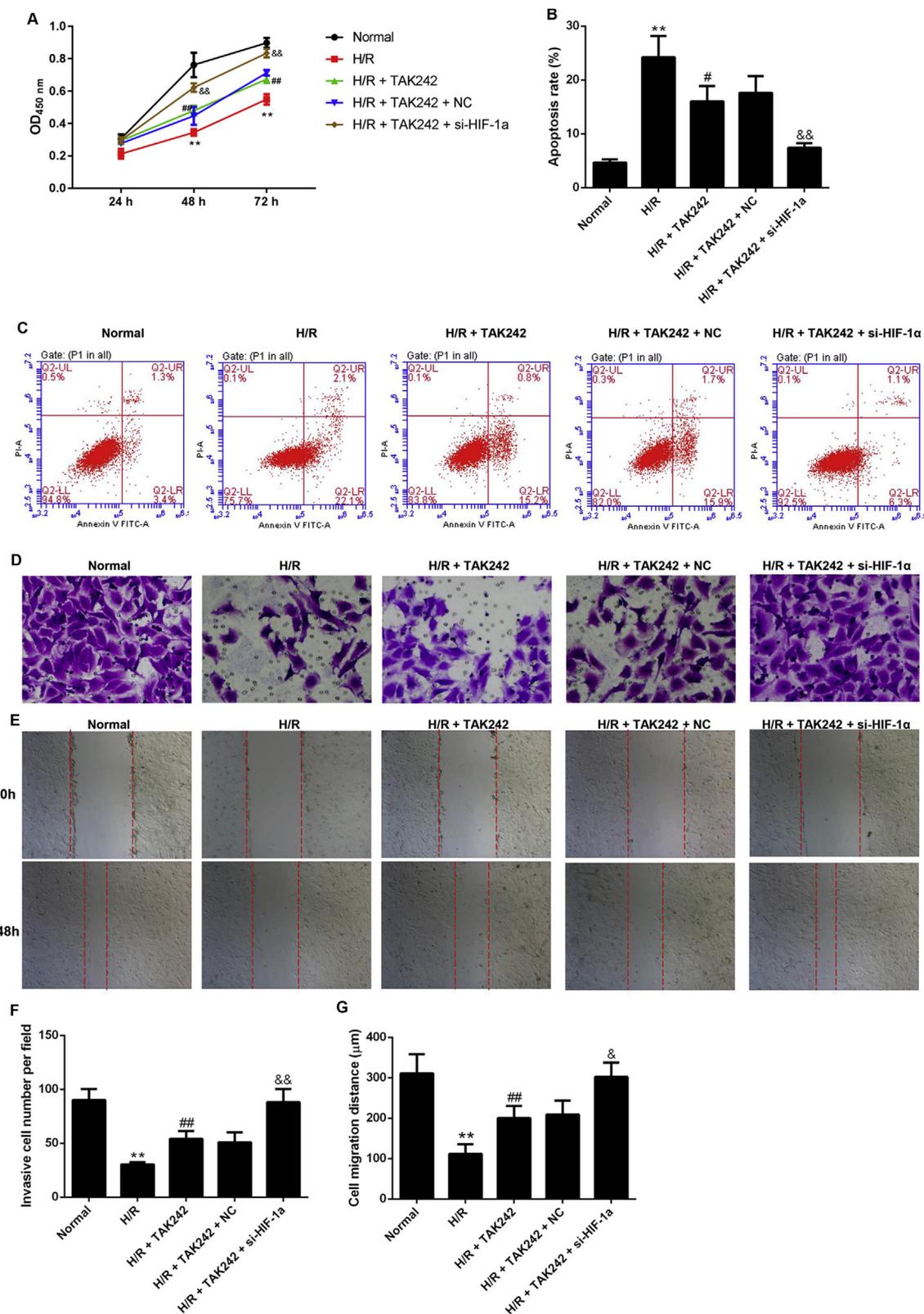


Fig. 4. Synergistic action of HIF-1a and TLR4 on hPMEC proliferation, apoptosis, migration, invasion in cellular PE model.

(A) Proliferation rates of hPMEC cells treated with TAK242 and HIF-1a siRNAs after hypoxia and reoxygenation. Cell proliferation rates were measured by the CCK-8 assay. (B) The apoptosis rates of hPMEC cells and cell apoptosis was determined by flow cytometry treated with TAK242 and HIF-1a siRNAs after hypoxia and reoxygenation; (C) Percentages of apoptotic hPMEC cells treated with TAK242 and HIF-1a siRNAs after hypoxia and reoxygenation; (D) Invasion potentials of hPMEC cells and cell number per field (F) treated with TAK242 and HIF-1a siRNAs after hypoxia and reoxygenation. Transwell system was applied to assess cell invasion. (E) Migration of hPMEC cells and cell migration distance (G) treated with TAK242 and HIF-1a siRNAs after hypoxia and reoxygenation treatment. Wound healing assay was performed to analyze cell migration ability. H/R: hypoxia and reoxygenation; TAK242: TLR4 inhibitor; HIF-1a: Hypoxia-inducible factor 1a; NC: negative control; siHIF-1a: siRNAs targeting HIF-1a; OD450: absorbance at 450 nm. **, ##, and && represented the significance between Normal and H/R group, H/R and H/R with TAK242 group, H/R with TAK242 and NC group and H/R with TAK242 and si-HIF-1a group, respectively. **, ##, and &&, P < 0.01.

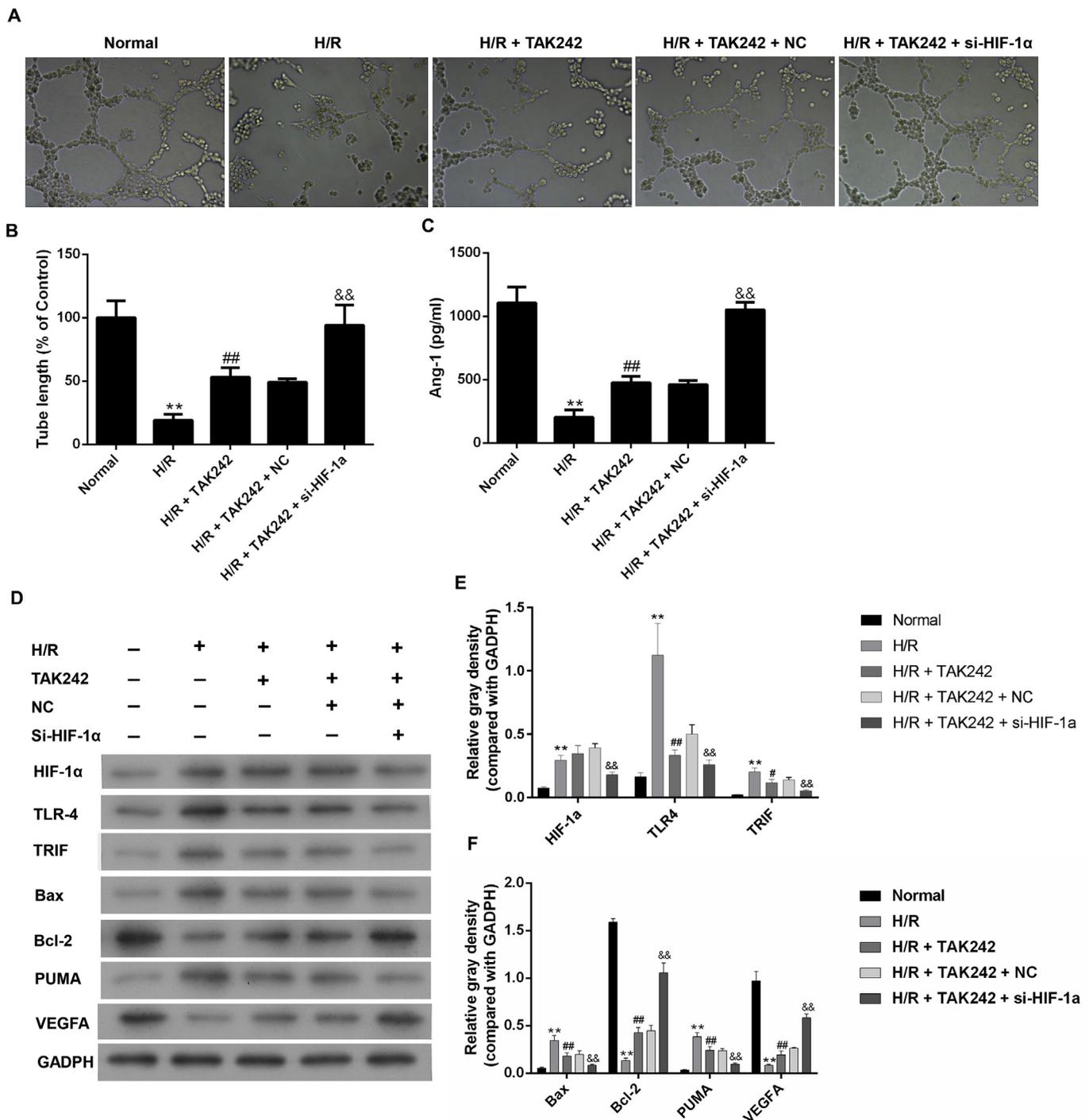


Fig. 5. Apoptosis and angiogenesis pathways regulated by HIF-1α and TLR4 in cellular PE model. (A) Angiogenic capability and tube length (B) of hPMEC cells treated with TAK242 and HIF-1α siRNAs after hypoxia and reoxygenation. Angiogenesis of hPMEC cells was analyzed by the tube formation assay. (C) Changes of ANG-1 levels in the culture supernatants of hPMEC cells treated with TAK242 and HIF-1α siRNAs after hypoxia and reoxygenation. ANG-1 concentrations in culture supernatants were measured by ELISA. (D) Abundances and relative gray density (E and F) of the HIF-1α, TLR4, TRIF, Bax, Bcl-2, PUMA and VEGF proteins in hPMEC cells with TAK242 and HIF-1α siRNAs after hypoxia and reoxygenation by western blotting. GAPDH was detected as the internal standard. H/R: hypoxia and reoxygenation; NC: negative control; siHIF-1α: siRNAs targeting HIF-1α; TAK242: TLR4 inhibitor; ANG-1: Angiopoietin-1; Bax: Bcl2-associated X protein; Bcl-2: B Cell Lymphoma 2; HIF-1α: Hypoxia-inducible factor 1α; TLR4: Toll-like receptor 4; TRIF: Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta; PUMA: P53 upregulated modulator of apoptosis; VEGFA: Vascular endothelial growth factor A; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. **, ##, and && represented the significance between Normal and H/R group, H/R and H/R with TAK242 group, H/R with TAK242 and NC group and H/R with TAK242 and si-HIF-1α group, respectively. **, ##, and &&, P < 0.01.

impairments of the proliferation, migration, invasion and angiogenesis capabilities of hPMEC cells, but significantly promoted cell apoptosis. However, the suppression of HIF-1α expression using specific siRNA transfection markedly reversed the above-mentioned cellular process

changes induced by hypoxia and reoxygenation in hPMEC cells, which supported the promoting roles of HIF-1α in H/R-induced damages of microvascular endothelial cells during PE development.

In addition, the expression of TLR4 in hPMEC cells was suppressed

by treatment with TAK242, also known as Resatorvid and act as a widely applied small molecule inhibitor of the TLR4 which selectively binds with TLR4 protein and inhibits the association of TLR4 with its adaptors [25]. The suppression of TLR4-related signaling pathways has been applied for previous investigation of TLR4 under contexts of various disorders such as acute cigarette smoke-induced pulmonary inflammation and liver ischemia and reperfusion injury [26,27]. In the present study, we showed that TAK242 treatment significantly enhanced the proliferation, migration, invasion and angiogenesis potentials of hPMEC cells after hypoxia and reoxygenation, and greatly repressed the H/R-induced hPMEC cell apoptosis. We demonstrated here that combination of silencing of HIF-1 α expression and inhibition of TLR-related signaling pathway exerted the synergistic effects on the cellular properties of hPMEC cells undergoing hypoxia and reoxygenation treatment.

For supporting the influences of HIF-1 α and TLR4 on the cellular function and angiogenesis of human placental vascular endothelial cells, the expression of several functional proteins associated with apoptosis and angiogenesis were further tested in hPMEC cells after H/R treatment and HIF-1 and TLR4 expression modulation. The expression of Bax and Bcl-2, two major mediators of cell apoptosis [28], were found to be oppositely altered in hPMEC cells by H/R treatments, and their alterations in cellular PE model were inhibited by HIF-1 α silencing and TLR4 inhibitor. Moreover, TRIF is a downstream player of the TLR-4 signaling pathway [29], and its expression was also greatly regulated in cellular PE model, but HIF-1 α silencing and TLR4 inhibitor also suppressed TLR-4 expressional changes induced by H/R. In addition, PUMA protein is a BH3-only Protein regulating the p53-mediated apoptosis in various cell types such as chronic lymphocytic leukemia cells [30]. We also proved here that PUMA expression was promoted by H/R treatment but suppressed by HIF-1 α siRNA and TLR4 inhibitors. Furthermore, the expression of angiogenesis-regulated protein VEGFA [31] also exhibited significantly changes in cellular PE model after HIF-1 α silencing and TLR4 inhibition, which is consistent with the changes of angiogenic capability of cellular PE model induced by HIF-1 α silencing and TLR4 inhibition. Moreover, ANG-1, an endothelial growth factor crucial for vascular development and angiogenesis [32] was also significantly increased in cellular PE model after silencing HIF-1 α and inhibiting TLR4. In summary, our analysis showed that HIF-1 α may suppress vascular endothelial cell proliferation, migration, invasion and angiogenesis but promotes cell apoptosis involved in TLR4 signaling during PE pathogenesis.

5. Conclusion

Silencing HIF-1 α and inhibiting TLR4 expression synergistically promoted cell proliferation, invasion, migration and angiogenesis but suppressed apoptosis in cellular model during PE pathogenesis.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.placenta.2019.06.375>.

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