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ORIGINAL ARTICLE

Triptolide inhibits angiogenesis in microvascular endothelial cells through regulation of miR-92a

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Abstract

Atherosclerosis is one common chronic inflammatory disease in which angiogenesis is involved. Here we established an in vitro cell model of angiogenesis made by human dermal microvascular endothelial cells (HMEC-1) and work to investigate the role of triptolide (TPL) in this model. To induce angiogenesis, HMEC-1 cells were cultured in Matrigel-conditioned medium. The ratio of tubes to nucleus was detected. To evaluate angiogenesis, Western blot assay was carried out to detect endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor receptor-2 (VEGFR2) and VEGF. Cell counting kit-8 was utilized to estimate the viability of HMEC-1 cells. microRNA (miR)-92a was analyzed by qRT-PCR. The targeting relationship between integrin subunit alpha 5 (ITGA5) and miR-92a was verified through luciferase activity assay. The effects of ITGA5 on signaling transducers (ERK, PI3K, and AKT) in a phosphorylated form were valued using Western blot method. After stimulated by TPL, LY294002 and PD98059, the alteration in phosphorylation of the signaling transducers was evaluated by Western blot assay. The ratio of tubes to nucleus and angiogenesis related factors were increased with the delaying of culture time. TPL decreased the expression of angiogenesis factors. Furthermore, miR-92a was upregulated by TPL and miR-92a silence upregulated angiogenesis factors. In addition, TPL decreased ITGA5 which was proved as a target of miR-92a. ITGA5 overexpression resulted in the abundance of angiogenesis factors while ITGA5 silence led to the opposite results. Meanwhile, ITGA5 overexpression increased phosphorylation of ERK, PI3K and AKT while ITGA5 silence reversed the trend. TPL (as an anti-angiogenesis agent) suppressed angiogenesis by upregulating miR-92a, and miR-92a-mediated down-regulation of ITGA5 blocked the signaling transduction of ERK and PI3K/AKT pathways.

Keywords Atherosclerosis · Angiogenesis · Triptolide · miR-92a · ERK · PI3K/AKT

Introduction

According to the World Health Organization global status report, cardiovascular diseases are responsible for the largest proportion of non-communicable diseases deaths under the age of 70 years [31]. The main etiology that contributes to cardiovascular diseases is basically atherosclerosis which clinically causes luminal narrowing, thrombi accumulation and cirulation obstruction [8, 26]. In this gradual progressive process, angiogenesis is initiated, and plaque instability may be ascribed to its incomplete maturation [19]. Currently, even though there are various kinds of approaches for treatment of atherosclerosis by regulating the levels of plasma high-density lipoprotein and low density lipoprotein [27], the long-term outcomes of atherosclerosis patients using these therapies are still poor. Besides, effective and novel therapeutic avenues are still urgently needed in the treatment of atherosclerosis.

Xiaomeng Xu and Li Tian contributed equally to this research.

Highlights
1. TPL inhibits HMEC-1 cell angiogenesis;
2. TPL upregulates the expression of miR-92a;
3. TPL inhibits cell angiogenesis via upregulating miR-92a expression;
4 ITGA5 is a target of miR-92a;
5. Overexpression of ITGA5 promotes angiogenesis while ITGA5 silence inhibits angiogenesis.

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Recently, traditional Chinese medicines receive considerable attention since a wide range of biological components possess the indispensable functions in multiple diseases, for instance, atherosclerosis [32]. Triptolide (TPL) is a naturally occurring component isolated from Chinese herb *Tripterygium wilfordii* Hook F., which possesses multiple biomedical properties, such as anti-inflammatory [35] and anti-cancer activities [10]. Actually, it has been confirmed that TPL halts the progression of atherosclerosis, not only based on its anti-inflammation effect, but also its modulatory function in lipid metabolism [18]. What's more, TPL, as a chemotherapeutic drug, exhibits splendid properties in precluding angiogenesis in mice melanoma [4]. Considering a large number of angiogenesis within the plaques leads to hemorrhage and thrombosis, which is closely related to the occurrence of cardiovascular and cerebrovascular events [13], we considered that the inhibitory effects might be due to its anti-angiogenic property.

On the other hand, as far as we are concerned, angiogenesis plays important roles in atherosclerosis during which process various microRNAs (miR) were involved [9, 28]. Of importance, miR-92a is identified as a potential marker of atherosclerosis [12]. Increasing evidence reveals that suppression of miR-92a accelerates fracture healing [21] and functional recovery of ischemic tissues [2]. The underlying mechanism is relatively associated with its anti-angiogenic activity [2, 14, 21]. It should be noticed that miR-92a antagonist and pre-miR-92a have been synthetized for modulation of angiogenesis [6, 7]. As a consequence, we considered that miR-92a might participate in the anti-angiogenic function of TPL in human dermal microvascular endothelial cells (HMEC-1). In other words, miR-92a might be the basis of its angiogenic activity.

Our study presented was designed to verify whether TPL possesses an ability to recede HMEC-1-dependent angiogenesis. Further, we tried to attest whether the anti-angiogenic property of TPL is dependent on the upregulation of miR-92a and downregulation of ITGA5. Finally, we dissected whether signaling transduction is involved.

Material and methods

Cell culture and treatment

HMEC-1 cells (ATCC, Rockville, MD, USA) were cultured in MCDB131 medium in addition with heat-inactivated fetal bovine serum (FBS; 10%) (Thermo Fisher Scientific, Waltham, MA, USA), L-glutamine (10 mM), hydrocortisone (1 μg/mL), epidermal growth factor (10 ng/mL), penicillin (100 units/mL) and streptomycin (100 μg/mL) (Sigma-Aldrich, St. Louis, MO, USA) and sustained in the humidified atmosphere containing 5% CO_2 and 95% air in 37 °C for 0–24 h. TPL at a purity of 98% (high performance liquid chromatography grade) (Sigma-Aldrich) was diluated in culture medium at different concentrations (0–5 μM). HMEC-1 cells were directly exposed to TPL at concentrations of 0–5 μM and incubated for 12 h. In accordance with a previous method [22], 25 μM LY294002 (PI3K inhibitor) and 50 μM PD98059 (ERK inhibitor) were applied to stimulate HMEC-1 cells for 30 min, respectively, to block ERK and PI3K/AKT signaling pathways.

Quantitative real time polymerase chain reaction (qRT-PCR)

For examining mRNA level, total RNA from HMEC-1 cells were collected using Trizol reagent (Invitrogen, Carlsbad, CA, USA). To detect integrin subunit alpha 5 (ITGA5), One Step SYBR® PrimeScript™ PLUS RT-PCR Kit (RR096; TaKaRa, Kyoto, Japan) was applied on Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument (Thermo Fisher Scientific) with PCR primers (forward primer, 5'-TGCCTCCCTCACCATCTTC-3′; reverse primer, 5'-TGCTTCTGCCAGTCCAGC-3′). As for miR-92a, TaqMan™ MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) was exploited with stem-loop RT primer. Next, real-time PCR was carried out on Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument. U6 was the internal control for miR-92a, and GAPDH was the internal control for ITGA5.

Generation of stably transfected cell lines

Short-hairpin RNA was ligated into U6/GFP/Neo plasmids (GenePharma, Shanghai, China) to silence ITGA5 by directly targeting it, with sh-NC as a negative control. To enforce ITGA5 overexpression, full-length of ITGA5 was fused into pEX-2 plasmid by GenePharma. Then, the stable transfected cells were cultured in selected culture medium conditioned with 0.5 mg/mL G-418 (Sigma-Aldrich). Similarly, miR-29a inhibitor was transduced into HMEC-1 cells by transfection, with NC inhibitor as a negative control (Invitrogen). Cell transfection was carried out in the presence of Lipofectamine 3000 reagent (Life Technologies, Carlsbad, CA, USA).

Cell viability assay

Cell counting kit-8 (CCK-8) assay was utilized to evaluate the viability of HMEC-1 cells as described in the manufacturer's protocol (Dojindo Molecular Technologies, Kumamoto, Japan). Firstly, HMEC-1 cells were seeded in a 96-well plate at a density of 5×10^3 cells per well for 12 h. Next, the cells were exposed to TPL and continually cultured for another 12 h. After that, 10 μL of CCK-8 solution was put into each well and then maintained for 1 h at an atmosphere containing 95% air and 5% CO_2 at 37 °C. Finally, the absorbance was

measured using Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific) at 450 nm.

Western blot

The whole cell lysate was extracted using RIPA lysis buffer (Beyotime, Shanghai, China) supplemented with protease inhibitors (Roche Applied Science, Indianapolis, USA). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The obtained protein was separated on a Bio-Rad Bis-Tris Gel system (Bio-Rad, Hercules, CA, USA) and then transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membranes were probed with primary antibodies which were prepared in 5% bovine serum albumin (BSA) (Thermo Fisher Scientific) at a dilution of 1:1000. The following primary antibodies were exploited for immunological analysis, including antibodies against endothelial nitric oxide synthase (eNOS) (ab5589), vascular endothelial growth factor receptor-2 (VEGFR2) (ab2349), vascular endothelial growth factor (VEGF) (ab53465), β-actin (ab8227), ITGA5 (ab150361), extracellular-signal-regulated kinase (ERK) 1/2 (ab17942), phospho (p)-ERK1/2 (ab214362), phosphatidylinositol 3′-kinase (PI3K) (ab180967), p-PI3K (ab182651), and protein kinase B (AKT) (ab131168) and p-AKT (ab38513), all purchased from Abcam (Cambridge, UK). After incubated with primary antibodies overnight at 4 °C, the membranes were washed and then the primary antibodies were hybridized by horseradish peroxidase (HRP)-linked secondary antibody (Abcam) at room temperature. After 1 h, the PVDF membranes were rinsed and transferred onto XRS system (Bio-Rad ChemiDoc™, Bio-Rad). The protein signals were visualized and digitized using Image Lab™ software (Bio-Rad) after the membrane was covered by 200 μL of Immobilon Western Chemiluminescent HRP Substrate (Millipore).

In vitro angiogenesis assay

To assess the formation of capillary tube-like structure, Matrigel-based assay was performed in this study. Matrigel was coated on the 6-well plate (BD Biosciences, CA, USA). Briefly, HMEC-1 cells (4×10^4 cells per well) were seeded into Matrigel extracellular matrix-conditioned medium and incubated for 24 h. Next, both cell number and tube-like cells were observed under a phase-contrast microscopy.

Dual luciferase activity assay

The complementary sequence of miR-92a was searched using TargetScan (www.targetscan.com) to predict the binding 3′-untranslated region (3'-UTR). Wild type 3'UTR of ITGA5 (ITGA5-wt) and mutant 3'UTR of ITGA5 (ITGA5-mut) were both synthesized by GenePharm (Shanghai, China). Next, the sequence of ITGA-wt and ITGA5-mut were cloned into pmirGLO Dual-luciferase expression vector from Promega, respectively. Next, the constructed plasmids and miR-92a mimic (with NC mimc as a negative control) were contransfected into HMEC-1 cells. Turner Biosystems 20/20 luminometer (Promega) was used to detect the activity of luciferase expressed by Dual-Luciferase Reporter Assay system (Promega).

Statistical analysis

All results are presented as the mean ± standard deviation (SD) of three repeated experiments. Statistical analyses were performed using GraphPad 6.0 statistical software (GraphPad, California, USA). The *P*-values were analyzed by student's *t* test and a one-way analysis of variance (ANOVA) with a post-hoc Tukey's test. *P*-values was considered to indicate a significant result when their values were less than 0.05.

Results

HMEC-1 cells was spontaneously vascularized in Matrigel culture

The tube-like structure of HMEC-1 cells were established when cells were seeded into Matrigel extracellular matrix [17]. Consistently the ratio of tubes to nucleus was evidently increased (12 h, $P < 0.05$; 24 h, $P < 0.01$, Fig. 1A). Besides, eNOS, VEGFR2 and VEGF were reported to exert vital roles in endothelial cell proliferation in the process of angiogenesis [3]. Additionally, as shown in Fig. 1B, eNOS, VEGFR2 and VEGF were statistically elevated in a time-dependent relationship ($P < 0.05$ or $P < 0.01$). The information above proved that HMEC-1-dependent angiogenesis spontaneously advanced in Matrigel extracellular matrix.

TPL inhibited the secretion of angiogenic mediators by HMEC-1

To prove the effect of TPL on production of angiogenic factors, we firstly set out to stimulate HMEC-1 cells with TPL at different concentrations and examine its direct role in cell viability. Our results suggested that TPL appeared to decrease cell viability when its concentration was equal or more than 4 μM ($P < 0.05$) but the inhibitory roles were not significant when its concentration was less than 4 μM ($P > 0.05$) (Fig. 2A). Hence, TPL at the concentration of 3 μM was used in the studies next. Further experiments were conducted to assess the potential functions of TPL on the crucial factors of angiogenesis: eNOS, VEGFR2 and VEGF. Interestingly, the expression of eNOS, VEGFR2 and VEGF was all down-regulated by TPL administration (all $P < 0.05$, Fig. 2B).

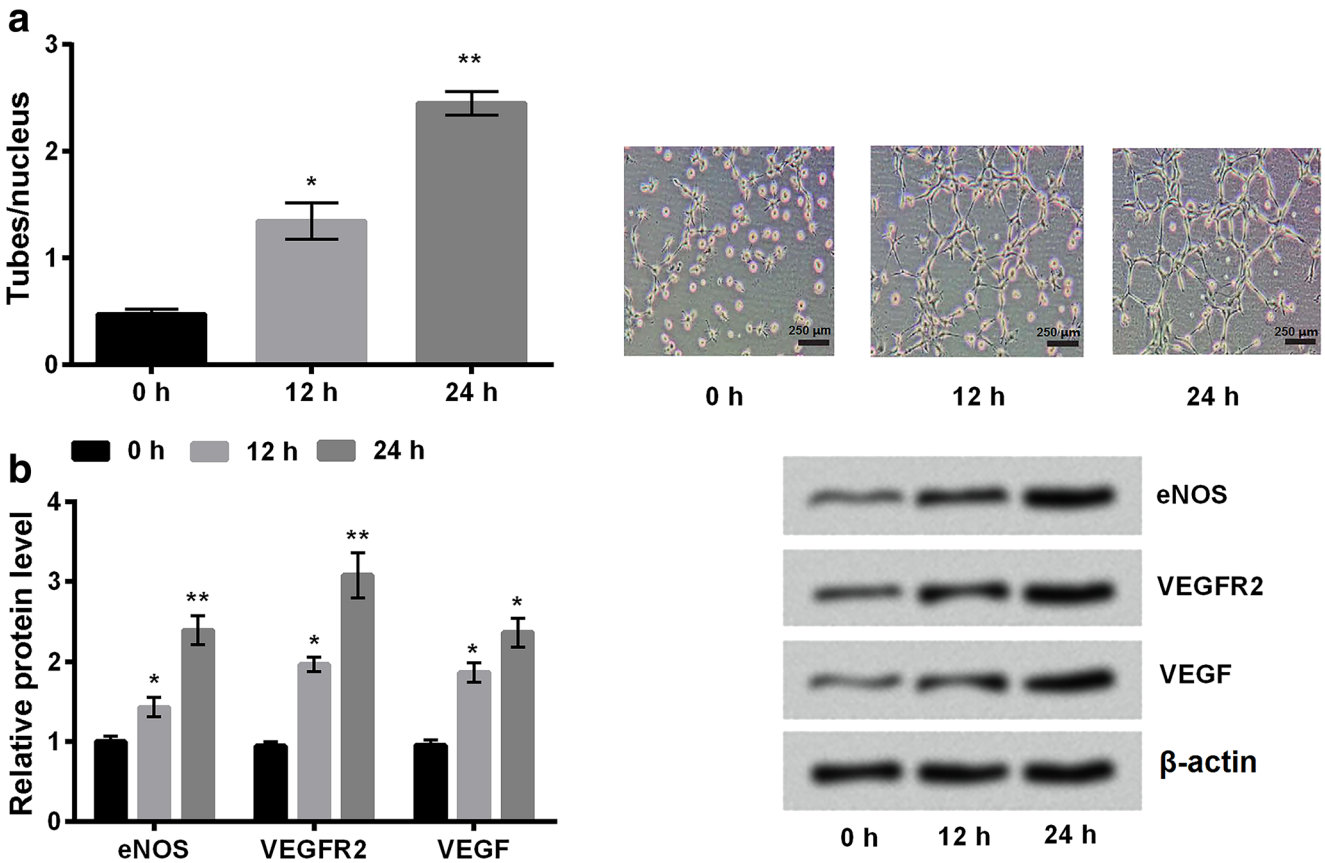

Fig. 1 Human microvascular endothelial cells (HMEC-1) were spontaneously vascularized in Matrigel-conditioned medium. (A) The ratio of tubes to nucleus was detected by angiogenesis assay. **(B)** Secretion of endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor receptor-2 (VEGFR2) and vascular endothelial growth factor (VEGF) was examined by Western blot. Data were presented as the mean ± SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$

TPL induced miR-92a expression

Several evidences showed miR-92a is closely involved in the progression of angiogenesis [2, 21]. Considering TPL brought considerable impact on angiogenesis, we speculated TPL might also influence miR-92a expression. Interestingly, we observed an altered production of miR-92a in HMEC-1 cells treated by TPL at concentrations of 2–3 μM ($P < 0.05$ or $P < 0.01$, Fig. 3), while there is no significance change of miR-92a in HMEC-1 cells administrated by 1 μM TPL. These results alluded to the idea that miR-92a might be related to the blockage of angiogenesis by TPL.

The production of angiogenic factors were inhibited by TPL-induced miR-92a

To identify an involvement of miR-92a in TPL-elicited downregulation of angiogenesis factors, miR-92a-deficiency HMEC-1 cells were constructed through transfecting miR-92a inhibitor into the cells. The apparent downregulation of miR-92a indicated high transfection efficiency ($P < 0.01$, Fig. 4A). As depicted in Fig. 4B, miR-92a silence significantly negated the suppressive effect of TPL on eNOS, VEGFR2 and VEGF compared with NC (all $P < 0.01$). In a word, we inferred that TPL might suppress angiogenesis through elevating miR-92a.

TPL impeded the generation of ITGA5 and ITGA5 emerged as a target of miR-92a

It has proved that miR-92a achieves its functions in angiogenesis through targeting a number of genes including ITGA5 [33]. Hence, we detected whether ITGA5 was also involved in the process. As expected, we found that TPL effectively downregulated the expression of ITGA5 ($P < 0.05$, Fig. 5A). In order to additionally confirm ITGA5 was a target of miR-92a during the progression, we constructed the specific binding sequence in 3'UTR of ITGA5 into pmirGLO dual-luciferase expression system and examined the activity of luciferase. Interestingly, the activity of luciferase was notably buffered in HMEC-1 cells simultaneously transfected with miR-92a mimic and ITGA5-wt. Collectively, there is a direct modulatory relationship between miR-92a and ITGA5.

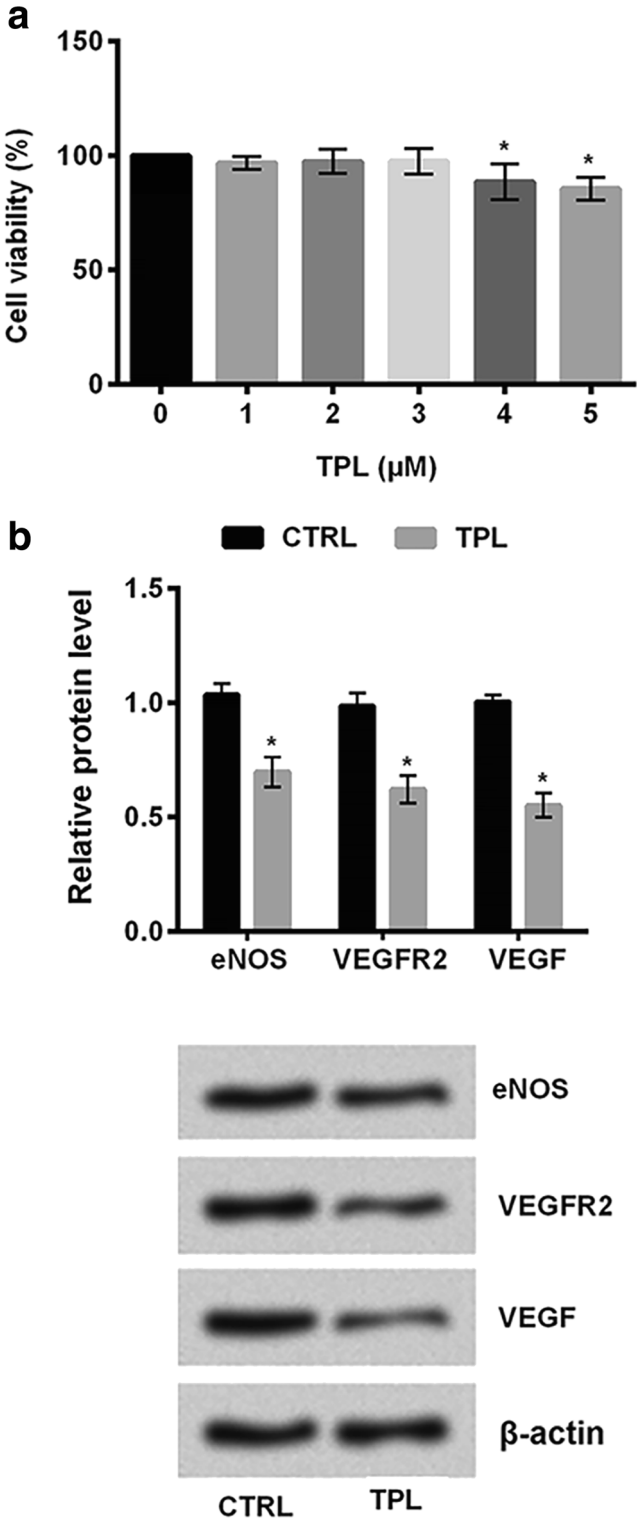

Fig. 2 Suppressive effect of triptolide (TPL) on secretion of endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor receptor-2 (VEGFR2) and vascular endothelial growth factor (VEGF) by human microvascular endothelial cells (HMEC-1). (A) HMEC-1 cells were exposed to TPL at the indicated concentrations, and their viability was detected by cell counting kit-8 (CCK-8) assay. **(B)** The expression of eNOS, VEGFR2 and VEGF was examined by Western blot. Data were presented as the mean ± SD ($n = 3$). *, $P < 0.05$

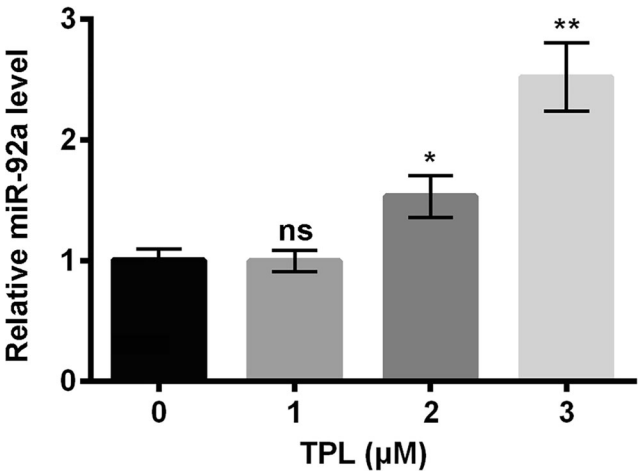

Fig. 3 Triptolide (TPL) increased the expression of microRNA-92a (miR-92a) in a dose-dependent manner. Human microvascular endothelial cells (HMEC-1) were incubated with TPL, and then miR-92a was detected by qRT-PCR. Data were presented as the mean ± SD (n = 3). ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$

ITGA5 promoted the accumulation of angiogenesis factors

In order to verify the functions of ITGA5, we transfected pEX-ITGA5 ($P < 0.01$) and sh-ITGA5 ($P < 0.01$) into HMEC-1 cells and found that they both had high transfection efficiency (Fig. 6A). In addition, we detected the expression of angiogenesis related factors. Notably, we found that overexpression of ITGA5 increased the accumulation of eNOS, VEGFR2 and VEGF ($P < 0.05$, $P < 0.01$ or $P < 0.001$), while ITGA5 silence notably controlled the abundance of the factors (all $P < 0.05$, Fig. 6B). Taken together, ITGA5 played an important role in the secretion of angiogenesis factors by HMEC-1.

ITGA5 triggered the signaling transduction of ERK and PI3K/AKT

A previous study reported that once ERK 1/2 and PI3K/AKT signal transduction is activated, angiogenesis is subsequently induced [5], suggesting that ERK1/2 and PI3K/AKT pathway is closely implicated in the angiogenesis process. Therefore, we explored whether ITGA5 is included in the modulation of phosphorylation of ERK1/2 and PI3K/AKT. As depicted in Fig. 7A-7B, ITGA5 overexpression obviously facilitated the phosphorylation of ERK1/2, PI3K and AKT compared with the control group (all $P < 0.001$). However, ITGA5 silence significantly counteracted the phosphorylation of ERK1/2, PI3K and AKT ($P < 0.05$ or $P < 0.01$, Fig. 7A-7B). Consequently, aberrant expression of ITGA5 was closely involved in regulating the signaling transduction of ERK1/2 and PI3K/AKT in HMEC-1 cells.

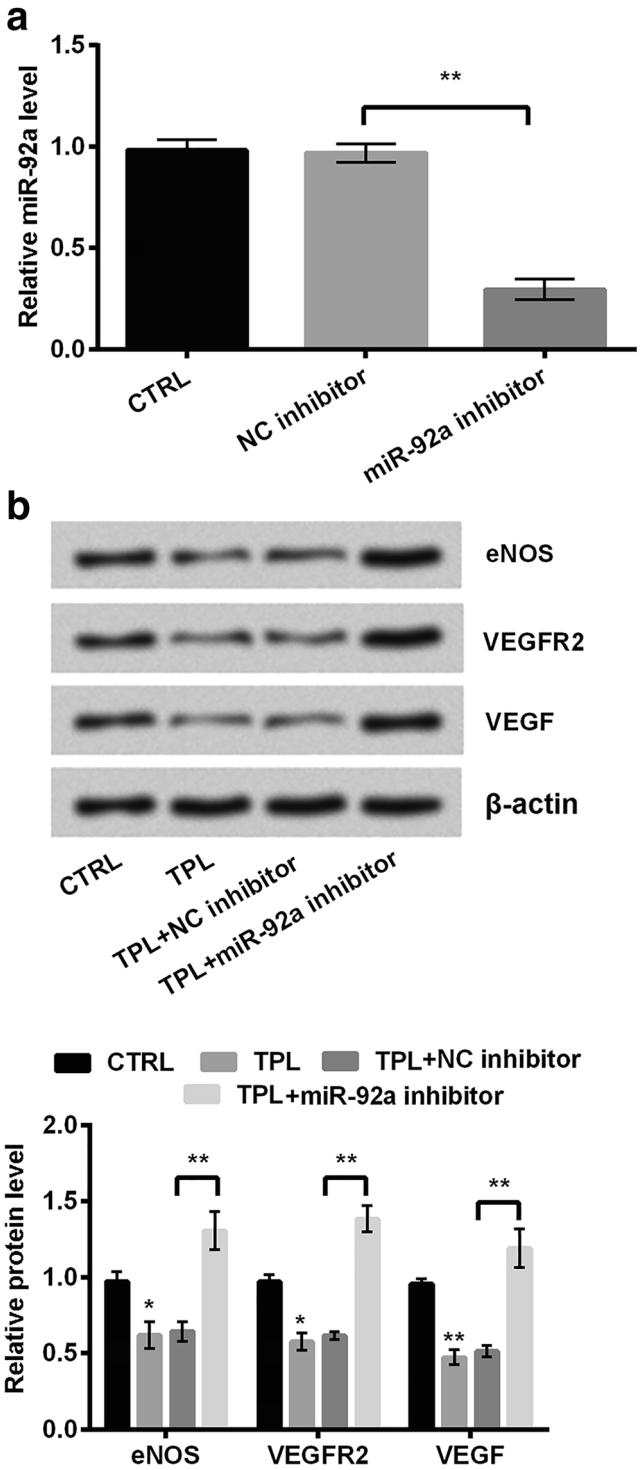

Fig. 4 Triptolide (TPL) suppressed the secretion of endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor receptor-2 (VEGFR2) and vascular endothelial growth factor (VEGF) through upregulation of microRNA-92a (miR-92a). (A) Human microvascular endothelial cells (HMEC-1) were transfected with miR-92a inhibitor, and transfection efficiency was identified by qRT-PCR. **(B)** After transfection, HMEC-1 cells were treated with TPL. The secretion of eNOS, VEGFR2 and VEGF by HMEC-1 was detected by Western blot. Data were presented as the mean ± SD (n = 3). *, $P < 0.05$; **, $P < 0.01$

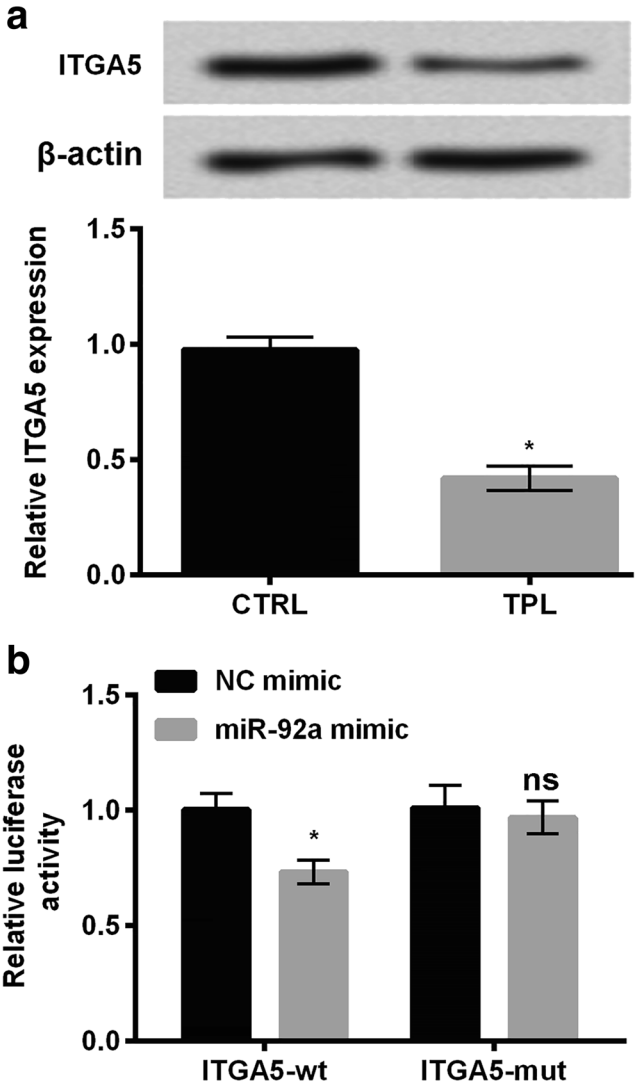

Fig. 5 microRNA-92a (miR-92a) functioned as a mediator of triptolide (TPL) in regulation of integrin subunit alpha 5 (ITGA5) in human microvascular endothelial cells (HMEC-1). (A) The effects of TPL on ITGA5 expression were detected by Western blot. **(B)** Luciferase activity was performed to confirm miR-92a directly targeting ITGA5. Data were presented as the mean ± SD (n = 3). ns, $P > 0.05$; *, $P < 0.05$

TPL showed a potential to block signaling transduction of ERK and PI3K/AKT pathways

Considering that ERK and PI3K/AKT are implicated in angiogenesis process, eNOS, VEGFR2 and VEGF might be downregulated once the signaling transduction was blocked. To prove this speculation, PI3K (LY294002) and ERK (PD98059) inhibitors were used to block PI3K/AKT and ERK signaling pathways. As inferred, protein levels of eNOS (both $P < 0.05$), VEGFR2 (both $P < 0.05$), and VEGF (both $P < 0.05$) were decreased in HMEC-1 cells treated by TPL, LY294002, and PD98059, respectively (Fig. 8A). In addition, ERK and PI3K/AKT pathways were continually

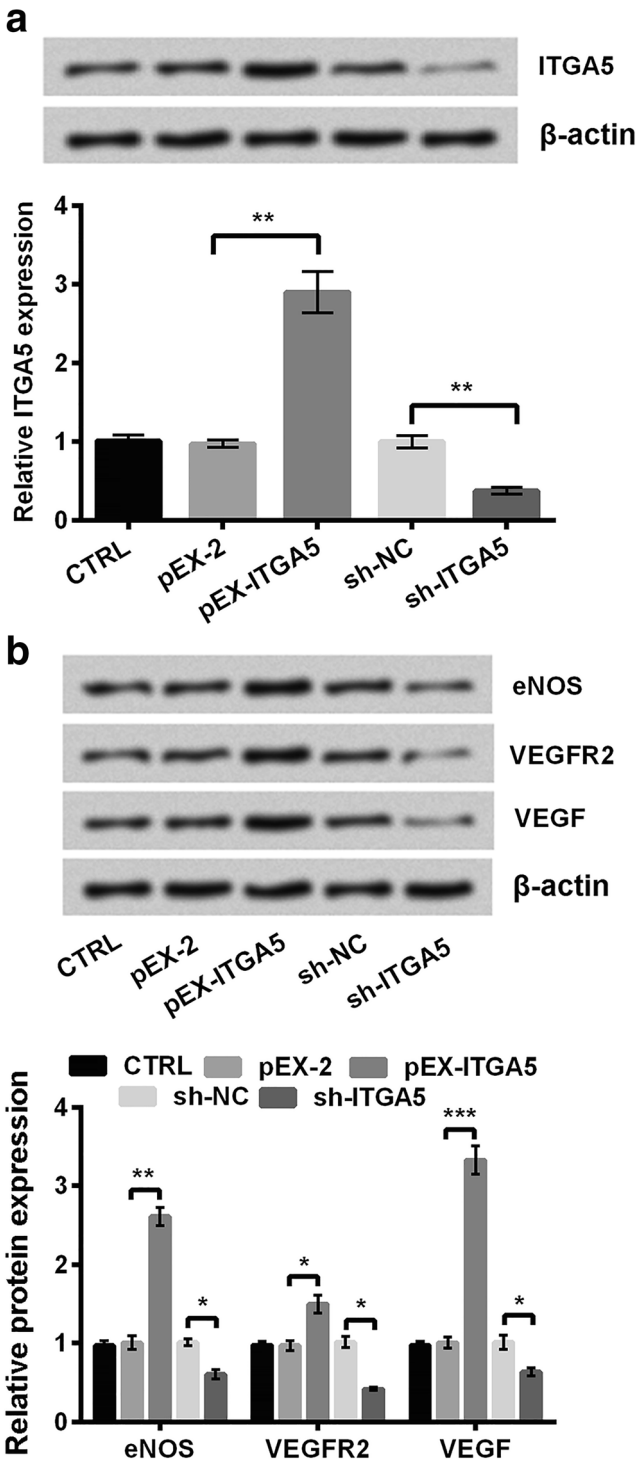

Fig. 6 Function of integrin subunit alpha 5 (ITGA5) on the secretion of endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor receptor-2 (VEGFR2) and vascular endothelial growth factor (VEGF) in human microvascular endothelial cells (HMEC-1). **(A)** HMEC-1 cells were transfected with the indicated plasmids in order to up-regulate and down-regulate ITGA5, and ITGA5 level was detected by Western blot (up) and qRT-PCR (down). **(B)** The expression of eNOS, VEGFR2 and VEGF was examined by Western blot. Data were presented as the mean ± SD (n = 3). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

examined. Both TPL ($P < 0.01$) and PD98059 ($P < 0.001$) blocked the phosphorylation of ERK instead of PI3K inhibitor (LY294002) ($P > 0.05$) (Fig. 8B). Relative to CTRL, TPL and PI3K inhibitor (LY294002) downregulated PI3K (both $P < 0.01$) and AKT (both $P < 0.01$) at phosphorylated levels, while a not significant alteration was observed in PD98059-treated cells (both $P > 0.05$) (Fig. 8C). Summarily, TPL possessed an anti-angiogenic capacity by acting on PI3K/AKT and ERK pathways.

Discussion

Nowadays, atherosclerosis results in the elevated disease prevalence and is the main etiology that leads to cardiovascular disease. The unhealthy life habit, such as sedentary lifestyle, unhealthy diets, smoking and excessive consumption of alcohol, is the main causing reason for atherosclerosis which has an extreme possible to be further translated into hypertension, diabetes, high blood lipid levels and altered permeability of the vascular endothelium [24]. Importantly, data from a previous study demonstrates that the dysregulation of endothelial cell viability, and metastasis, especially cell angiogenesis bring considerable impacts in endothelial function, which exerts crucial effects on the progression of atherosclerosis [Gimbrone and Garcia-Cardena, 2016].

The cell model of angiogenesis is often established by using HMEC-1 cells [20]. Hence, we used HMEC-1 cells to construct cell model of angiogenesis. By using Matrigel-conditioned culture, the ratio of tubes to nucleus was visibly elevated in a time-dependent manner. This indicated that HMEC-1 cells were spontaneously vascularized. In addition, we detected the expression of angiogenic factors, including eNOS, VEGFR2 and VEGF, which are important angiogenesis-related factors in the process of angiogenesis [11]. With the passage of time, the expression of eNOS, VEGFR2 and VEGF were upregulated, suggesting that the cell model was successfully established.

TPL is generally extracted from the Chinese herb *Tripterygium wilfordii* Hook F. which is often used in various kinds of diseases [29, 34]. Of notably, TPL has been found to exert an inhibitory effect in atherosclerosis in mice [18]. Therefore, we worked to prove whether TPL possesses a potent to suppress angiogenesis. Results from our study proved that TPL under the concentration of 3 μM did not influence cell viability but significantly downregulated the expression of angiogenesis-related factors, including eNOS, VEGFR2 and VEGF. This result revealed the potential effects of TPL in alleviating angiogenesis in HMEC-1 cells, which suggested that TPL possesses functions in suppressing atherosclerosis.

Further experiments were performed to explore the possible mechanism. It is well-known that miRs participate in almost all of the biological activities, including atherosclerosis

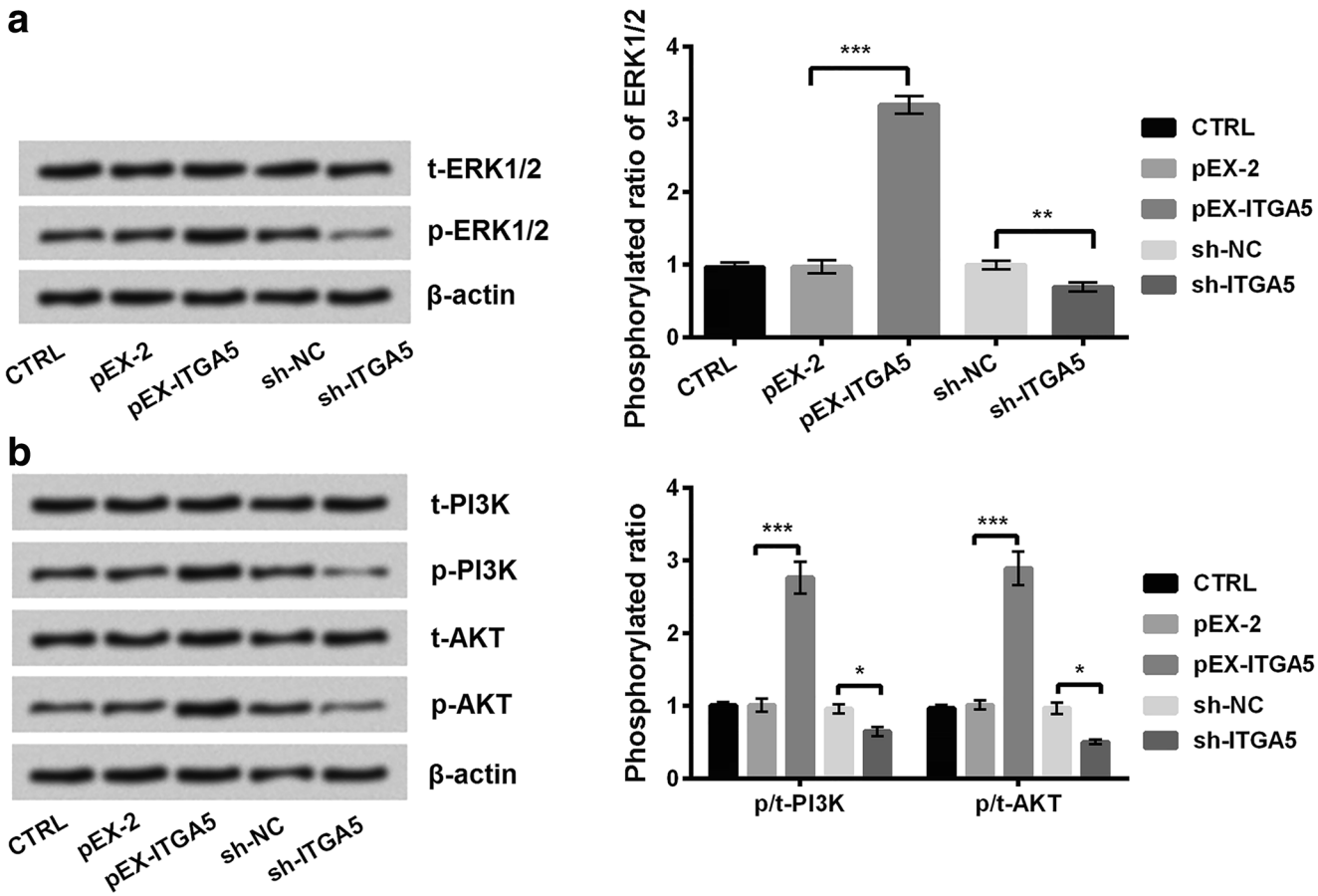

Fig. 7 Role of integrin subunit alpha 5 (ITGA5) on extracellular-signal-regulated kinase (ERK) and phosphatidylinositol 3′-kinase/protein kinase B (PI3K/AKT) signal pathways in human microvascular endothelial cells (HMEC-1). The phosphorylated ratio of **(A)** ERK1/2, **(B)** PI3K and AKT was detected by Western blot. Data were presented as the mean ± SD (n = 3). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

[1]. For instance, miR-155 enhances atherosclerosis via inhibiting Bcl-6 in macrophages [23]. miR-342-5p has been revealed to function in promoting inflammatory macrophage activation within the process of atherosclerosis [30]. Among all these identified miRs associated with atherosclerosis, miR-92a has an activity in increasing endothelial activation, decreasing plaque stability and promoting atherosclerosis [16]. However, there is a paucity of data on the effects of miR-92a on angiogenesis during atherosclerosis, as well as it was regulated by the active compound from traditional Chinese medicine.

Firstly, we found that miR-92a was upregulated by TPL in a dose-dependent way, which indicated miR-92a might be involved in this regulation progression. Further experiments confirmed this inference. Treatment with TPL in miR-92a-deficiency HMEC-1 cells increased the accumulated levels of all these three angiogenesis-related factors eNOS, VEGFR2 and VEGF, which proved that TPL decreased angiogenesis through upregulation of miR-92a. Through this approach, it is also demonstrated that miR-92a inhibited angiogenesis and then inhibited atherosclerosis. By contrast, results from one previous study revealed that miR-92a promotes atherosclerosis [16]. Consistent with our studies, a previous study found miR-92a inhibits angiogenesis in vitro and in vivo [2]. These different pathogenesis mechanisms might be responsible for this discrepancy.

In general, miRs often achieves their functions through targeting some other genes. In an animal model of ovarian cancer, ITGA5 has been ascertained as a target of miR-92a [25]. In our study, we also confirmed that conclusion in HMEC-1 cells, that is to say, ITGA5 was a direct target of miR-92a. In addition, TPL significantly enhanced the expression of ITGA5. Thereafter, we found that ITGA5 overexpression increased the accumulated levels of eNOS, VEGFR2 and VEGF while ITGA5 silence brought out downregulation of these factors. These results demonstrated that TPL could upregulate miR-92a which modulated ITGA5 and finally led to an alteration in the expression of eNOS, VEGFR2 and VEGF. Our results were in line with the previous study that miR-92a targets ITGA5 in the process of angiogenesis [33].

In the end, we also analyzed the possible pathways which involved in atherosclerosis. ERK and PI3K/AKT were two

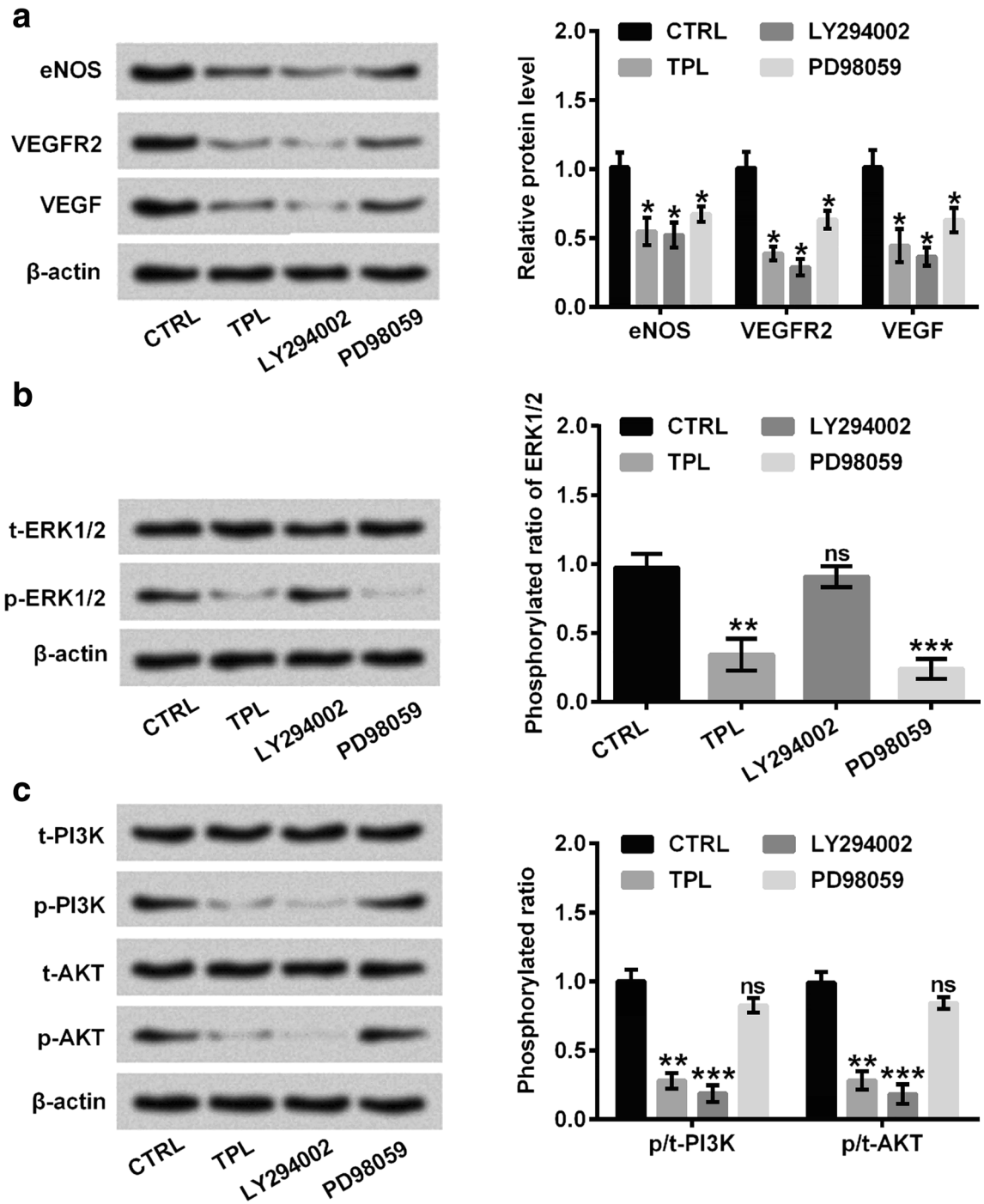

Fig. 8 Effects of triptolide (TPL) and signaling inhibitors for phosphatidylinositol 3′-kinase (PI3K) and extracellular-signal-regulated kinase (ERK) on the expression of endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor receptor-2 (VEGFR2) and vascular endothelial growth factor (VEGF) as well as the activation of signaling pathways. HMEC-1 cells were stimulated with 3 μM TPL for 12 h, and treated with 25 μM LY294002 or 50 μM PD98059 for 30 min, respectively. Western blot assay was carried out to examine **(A)** eNOS, VEGFR2, VEGF, **(B)** ERK1/2, **(C)** PI3K and protein kinase B (AKT). Data were presented as the mean ± SD (n = 3). ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

important pathways in atherosclerosis and were normally activated in the process of angiogenesis [15]. Consistently, we found ITGA5 overexpression visibly strengthened the phosphorylation of key regulators of ERK and PI3K/AKT pathways, while the phosphorylation was blunted by ITGA5 silence. Combined with the former results, these findings confirmed that overexpression of ITGA5 promoted angiogenesis and triggered the activation of ERK and PI3K/AKT cascades, which was consistent with former studies [15]. Besides, we confirmed that TPL and signaling inhibitors (LY294002 and

PD98059) blocked the angiogenesis by acting on eNOS, VEGFR2 and VEGF. Continually, we compared the inhibitory role of TPL on signaling pathways with that of signaling inhibitors (LY294002 and PD98059). We noticed that TPL effectively precluded signaling transduction of ERK and PI3K/AKT cascades.

Collectively, TPL repressed the secretion of angiogenesis factors by HMEC-1 cells. Then we found that miR-92a was a crucial mediator of TPL in inhibiting angiogenesis factors accumulation. Moreover, through upregulating miR-92a, TPL repressed the production of ITGA5 which was directly targeted by miR-92a. Overexpression of ITGA5 promoted angiogenesis and also activated ERK and PI3K/AKT pathways. Our study unveiled the underlying mechanism about the function of TPL on HMEC-1 cells.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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