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Resveratrol modulates angiogenesis through the GSK3β/β-catenin/TCF-dependent pathway in human endothelial cells

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ABSTRACT

Vascular endothelial growth factor (VEGF) plays a critical role in angiogenesis due to its potent and specific ability to promote the proliferation and migration of endothelial cells. Resveratrol has been shown to have many health-benefiting effects, including the protection of cardiovascular system. In this study we examined the effect of resveratrol on angiogenesis in human umbilical vein endothelial cells (HUVECs). We observed that resveratrol was able to modulate the expression of *VEGF* and the formation of vascular network in a biphasic pattern. While resveratrol at low concentrations, from 1 to 10 μM, up-regulated the expression of *VEGF* and promoted angiogenesis, it had opposite effect at high concentrations (20 μM and higher). The biphasic effect of resveratrol on angiogenesis was confirmed by chick chorioallantoic membrane assay. Up-regulation of *VEGF* expression depended on the nuclear accumulation and transcriptional activity of β-catenin. Correspondingly, GSK3β, a negative regulator of β-catenin, turned into a less active state (phosphorylated at Ser9) in cells exposed to 5 μM of resveratrol, but became more active at 20 μM. We demonstrated that both Akt and ERK signaling pathways, which are known to be critical for angiogenesis, became activated in response to 5 μM of resveratrol and functioned to inactivate GSK3β. Our findings may have implications in the management of cardiovascular diseases and other conditions such as cancer by the use of resveratrol.

1. Introduction

Angiogenesis, the sprouting of new capillaries from the preexisting vasculature, is integral to many physiological and pathological processes, including embryonic vascular development, wound healing, organ regeneration, diabetic retinopathy, rheumatoid arthritis, cardiovascular diseases, tumor growth and metastasis [1]. During angiogenesis, endothelial cells proliferate, migrate, and form tube-like structures. This cascade of events is tightly controlled by angiogenic factors. Vascular endothelial growth factor (VEGF) has potent and specific ability to regulate key steps in angiogenesis, including proliferation and migration of endothelial cells [1]. Over-expression of VEGF and its receptors promotes blood vessel formation, whereas the inhibition of VEGF function blocks angiogenesis [1,2].

Glycogen-synthase kinase 3β (GSK3β) serves as a nodal point of convergent signaling pathways in endothelial cells in regulating angiogenic responses. Inhibition of GSK3β induces an angiogenic phenotype in endothelial cells [3]. GSK3β is known to be regulated by several signaling pathways, including PI3K/Akt [4,5], MEK/ERK [3,6], and Wnt signaling pathway [7]. Among them the Wnt/GSK3β pathway is most extensively studied. In the presence of Wnt signaling, CKI and GSK3β become inactivated, leading to cytoplasmic, and subsequently nuclear, accumulation of β-catenin. β-catenin in the nucleus forms complexes with members of the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors to activate transcription [8,9]. In the absence of Wnt signaling, β-catenin is constitutively phosphorylated, by GSK3β and CKI, at serine and threonine residues in the N-terminal region, resulting in ubiquitination and subsequent proteosomal degradation [9,10]. Increasing evidence has implicated the Wnt/β-catenin signaling pathway in vascular development in normal and pathological conditions [11,12]. Wnt ligands and their receptors are expressed in vascular cells [11]. Endothelial cell-specific inactivation of the β-catenin gene in mice results in defective vascularization and embryonic lethality [12].

Several studies showed that the human *VEGF* promoter contains β-catenin-TCF binding motifs and that *VEGF* promoter activity can be increased by stabilized β-catenin [5,13,14]. Moreover, β-catenin is sufficient to promote vessel growth in vivo and confer a pro-angiogenic phenotype to endothelial cells in vitro through the

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transcriptional activation of *VEGF* [14]. In addition, *VEGF* expression can also been stimulated by hypoxia inducible factor (HIF-1) [1]. Therefore, VEGF serves as an important target molecule in treating diseases resulting from insufficient or excessive blood vessel formation.

Increasing evidence has shown that resveratrol (trans-3,5,4′-trihydroxystilbene) possesses anti-oxidant, anti-cancer, anti-aging, and anti-inflammatory function. In particular, the cardioprotective effect of resveratrol has been intensively investigated [15]. Resveratrol has been found to protect the vessels from atherosclerosis [16], to reduce myocardial damage during ischemia-reperfusion [17,18], and to modulate vascular cell functions [19]. Nitric oxide (NO) [20], thioredoxin-1 [19], adenosine receptors [21,22], PI3K and mitogen-activated protein kinase (MAPK) [17,23], and mTOR [18] have all been proposed to mediate the cardioprotective effects of resveratrol.

Resveratrol can activate both the PI3K/Akt and the MAPK pathways [17,23], which negatively regulate GSK3β. We previously showed that resveratrol could promote osteoblastic differentiation by augmenting β-catenin signaling pathway [24]. In this study we examined the effect of resveratrol on angiogenesis. We observed that resveratrol modulates angiogenesis in a biphasic pattern. Whereas low concentration of resveratrol promotes angiogenesis in human umbilical vein endothelial cells (HUVECs), at high concentration it has inhibitory effect. We demonstrated that resveratrol modulates angiogenesis in HUVECs through GSK3β/β-catenin/TCF pathways, which is in turn regulated by the activation of PI3K/Akt and MEK/ERK signaling pathways.

2. Materials and methods

2.1. Experimental reagents

Resveratrol (Sigma, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the culture medium was less than 0.05% (v/v). Control cultures received the same amount of DMSO. Dulbecco's Minimum Essential Medium (DMEM), fetal bovine serum, lithium chloride (LiCl) were purchased from Gibco (Carlsbad, CA, USA). The PI3K inhibitor, LY294002, the MEK1 inhibitor, PD98059, the polyclonal phospho-specific antibodies against Akt (Ser473), ERK/1/2, GSK3β (Ser9) and β-catenin (Ser33/37/Thr41) and monoclonal anti-Akt, ERK and GSK3β were from Cell Signaling Technology (Beverly, MA, USA). Anti-β-actin, anti-histone-H2A, anti-β-catenin and anti-VEGF-A monoclonal antibodies were from Santa Cruz (Santa Cruz, CA, USA). Lipofectamine 2000, and RNA isolation reagent TRIZOL were products of Invitrogen (Carlsbad, CA, USA). TOPFLASH and FOPFLASH constructs were from Upstate biotechnology (Lake Placid, NY). PGL3-basic Vector, dual luciferase reporter assay kit and AMV reverse transcriptase were obtained from Promega (Madison, WI, USA). Cell lysis buffer for Western were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). NE-PER nuclear and cytoplasmic extraction reagents were from Pierce (New York, NY, USA). PVDF membrane and ECL PLUS were from GE Healthcare (Princeton, NJ, USA). Growth factor-reduced matrigel was obtained from BD Biosciences (Bedford MA, USA). Anti-human VEGF neutralizing antibodies (catalog number MAB293 mentioned as VEGF-Ab) were purchased from R&D Systems (Minneapolis, MN, USA). Small interfering RNA (siRNA) oligonucleotide targeting β-catenin and a negative control were obtained from Shanghai Gene Pharma Co., Ltd (Shanghai, China).

2.2. Cell culture and treatments

HUVECs were purchased from ATCC and cultured in DMEM supplemented with 10% fetal bovine serum, 0.1 mg/ml heparin, and 1% penicillin/streptomycin in a humidified incubator at 37 °C with 5% CO_2. For positive control, 10 mM LiCl was added in DMEM. For inhibition assays, HUVECs were pretreated with 100 μM PD98059 or 20 μM LY294002 for 1 h prior to the addition of resveratrol.

Primary endothelial cells (EC) were isolated from human umbilical veins and cultured essentially as described previously [25]. The cells were cultured in Medium 199 with Earle's salts and L-glutamine and supplemented with 15% fetal bovine serum and 1% penicillin/streptomycin. The cells exhibited typical cobblestone morphology and were used between passages 1 and 4. Endothelial cell identity was confirmed by the presence of Factor VIII related antigen. The cells were treated with resveratrol at different concentrations and VEGF-Ab for endothelial tube formation assay.

2.3. Western blot analysis

HUVECs were washed with PBS and lysed with passive lysis buffer. Cytoplasmic and nuclear fractions were prepared using the NE-PER nuclear and cytoplasmic extraction reagents according to the manufacturer's protocol. Protein concentration was determined by BCA Protein Assay with BSA as the standard. Equal amounts of protein were subjected to SDS–PAGE and electro-transferred to a PVDF membrane. After blocking with 5% skimmed milk, blots were probed with primary antibodies at 4 °C overnight. After washing thrice with TBS-T, the membranes were treated with horseradish peroxidase-conjugated secondary antibodies (dilution 1:10,000) for 1 h and visualized by ECL PLUS.

2.4. Quantitative reverse-transcription PCR

Total RNAs from HUVECs were extracted with TRIZOL reagents according to the manufacturer's protocol. Any potential DNA contamination was removed by RNase-free DNase treatment. cDNA was synthesized from 1 μg of total RNA by AMV reverse transcriptase. The primers for human VEGF were 5′-TGC CTT GCT GCT CTA CCT CC-3′ (forward) and 5′-GAT TCT GCC CTC CTC CTT CT-3′ (reverse), primers for human VEGFR1 were 5′-AAC AGC AGG TGC TTG AAA CC-3′ (forward) and 5′-TCG CAG GTA ACC CAT CTT TTA AC-3′ (reverse), primers for human VEGFR2 were 5′-AGT GAT CGG AAA TGA CAC TGG A-3′ (forward) and 5′-GCA CAA AGT GAC ACG TTG AGA T-3′ (reverse), and primers for β-actin were 5′-GTT GCG TTA CAC CCT TTC TTG-3′ (forward) and 5′-CTG CTG TCA CCT TCA CCG TTC-3′ (reverse). Real time PCR was done using a SYBR green PCR mix (Applied Biosystems) in an ABI 7500 Sequence Detection System (Applied Biosystems).

2.5. Immunofluorescence

HUVECs, grown on coverslips in the 24-well plates, were washed in PBS and then fixed by 4% paraformaldehyde for 20 min at room temperature, and then permeabilized using 0.1% Triton-X100 (Sigma) in PBS for 20 min. Cells were blocked with 5% normal goat serum in PBS for 1 h, followed by mouse anti-β-catenin monoclonal antibody (1:200) and rhodamine-conjugated anti-mouse secondary antibody (Jingmei). After washing with PBS thrice, cells were further stained with 4, 6-diamidino-2-phenylindole (DAPI) for 10 min. The coverslips were rinsed in PBS and water and then mounted on glass slides with antifade mounting medium. The fluorescence signal was examined with a DP71 fluorescence microscope (Olympus).

2.6. TOPFLASH luciferase reporter assay

In 48-well plates, cells were transiently transfected with 0.2 μg of the TOPFLASH or FOPFLASH reporter plasmid using 1 μl

Lipofectamine (Invitrogen). As a control for transfection efficiency, 0.02 μg of pRL-TK vector that provided constitutive expression of Renilla luciferase was included in each transfection. Six hours after transfection, cells were treated with resveratrol (0, 5 μM) or LiCl for 24 h. Then the cells were harvested and extracts were prepared in 50 μl of passive lysis buffer (Promega). The luciferase activity was determined with the use of Perkin-Elmer 1420 multilabel counter according to the instruction of the Dual-Luciferase Reporter® assay system (Promega). All experiments were carried out in triplicate.

2.7. VEGF reporter construction, transient transfection and luciferase assay

DNA fragments of human *VEGF* promoter (−351 bp to +32 bp) containing the TCF/LEF response elements were generated by PCR with the use of reverse primer (5′-CCC AAG CTT ACG ACC TCC GAG CTA CCC-3′) and the following sense primers that are flanked by Kpn I and Hind III restriction sites: for construct WT which contains TCF response element: 5′-CGG GGT ACC AGC AAA GAG GGA ACG GCT-3′; for construct mTCF which contains two point mutations in the core sequence of TCF response element: 5′-CGG GGT ACC AGC GAG GAG GGA ACG GCT-3′. Cycling conditions included an initial denaturation at 94 °C for 5 min followed by 35 cycles at 94 °C for 35 s, 58 °C for 35 s, 72 °C for 35 s, and a final extension at 72 °C for 10 min. The products were gel-purified and fused to promoterless luciferase reporter plasmid PGL3-basic. The sequences of all constructs were confirmed by restriction digestion and sequencing.

2.8. RNA interference of β-catenin

Two siRNA oligonucleotides targeting β-catenin with sequences of 5′-GCA GUU GUA AAC UUG AUU ATT-3′ (forward) and 5′-UAA UCA AGU UUA CAA CUG CTT-3′ (reverse) and a negative control were transfected into HUVECs. Cells were transfected with β-catenin-siRNA or nonspecific siRNA and post-treated with or without resveratrol for 24 h. Western blot was performed using VEGF antibody. All test samples were performed in triplicate.

2.9. Endothelial tube formation assay

Plates (48 well) were coated with 200 μl growth factor-reduced Matrigel and incubated at 37 °C for 1 h to allow gelling. HUVECs (4×10^4 cells/well) were seeded on matrigel-coated plates. And then HUVECs were untreated or treated with resveratrol, LiCl, a combination of resveratrol and VEGF-Ab or various inhibitors for 24 h at 37 °C. The number of tubes was counted using a phase contrast microscopy (×10). Twelve microscopic fields were randomly selected for each well, and the number of tube-like structures per field was counted. All test samples were performed in triplicate.

2.10. Chick chorioallantoic membrane (CAM) assay

CAM assay was performed as described previously [26]. Fertilized chick eggs were incubated for 7 days at 37 °C and relative humidity of 80%. After incubation, eggs were opened on the air sac side and chick embryos were prepared by separating the CAM from the shell membrane. Filter disks with various test substances were placed on the CAM. The cavity was covered with parafilm, and eggs were incubated for additional 72 h and CAMs were removed for analysis [27]. Angiogenesis was quantified by counting the number of branching blood vessels. Each experiment was performed three times.

2.11. Statistical analysis

Data were expressed as the mean ± SEM based on at least three independent experiments. Analysis of variance (ANOVA) with a one-tailed Student's *t* test was used to identify significant differences in multiple comparisons. Probability values were considered significant at $P < 0.05$.

3. Results

3.1. Resveratrol modulates VEGF and VEGFR2 expression in a biphasic manner

Because VEGF is critical in angiogenesis, we first examined the transcription of *VEGF* in HUVECs in response to resveratrol. HUVECs were treated with increasing concentrations of resveratrol (0.2, 1, 5, 10, 15, 20, and 50 μM) for 24 h, the mRNA level of *VEGF-A* was determined by using quantitative real-time PCR. As shown in Fig. 1A, resveratrol treatment changed the expression levels of *VEGF* in a dose-dependent manner. When applied in the range from 1 to 10 μM, resveratrol significantly increased *VEGF* expression, whereas at higher concentration, 20 and 50 μM, it inhibited the transcription of *VEGF*. While *VEGF* transcription peaked at 5 μM, a sharp drop was observed at 15 μM. In addition, the stimulatory effect of resveratrol on *VEGF* expression was time-dependent. Elevated levels of *VEGF* mRNA were evident at 24 h, and become more pronounced at 48 h after resveratrol (5 μM) was applied (Fig. 1B). Western blot analysis confirmed the change of *VEGF* expression at protein level. The levels of VEGF protein increased when cells were exposed to 1 μM, peaked at 5 μM, significantly decreased at 20 and 50 μM (Fig. 1C). VEGF protein was also significantly increased at 24 h and become more evident at 48 h (Fig. 1D). These results indicate that resveratrol can regulate VEGF expression in a biphasic pattern. Resveratrol at low concentrations stimulated the expression of VEGF, but inhibited its expression at higher concentrations. We chose 5 and 20 μM to investigate the possible mechanisms by which resveratrol modulates angiogenesis.

VEGF transmits angiogenic signals through VEGF receptors (VEGFR). We next examined the expression of *VEGFR* in HUVECs in response to resveratrol. In accordance with the VEGF induction results, while resveratrol at 5 μM significantly upregulated *VEGFR2* mRNA expression, it had inhibitory effect at 20 μM. In contrast, the mRNA levels of *VEGFR1* remained unaffected (Fig. 1E and F).

3.2. Resveratrol modulated angiogenesis

We used a vascular network formation assay to examine whether the resveratrol-induced changes in VEGF levels correspond to change in the angiogenic activity of HUVECs. In this assay, Matrigel serves as a matrix for endothelial cells to migrate and align in the formation of network structures. This assay mimics the multistep process of angiogenesis involving cell adhesion, migration, differentiation, and growth [5]. As shown in Fig. 2A, 5 μM resveratrol caused a significant increase in tube formation. Consistent with the *VEGF* expression data, the effect of resveratrol on tube formation was concentration-dependent. 5 μM resveratrol promoted network formation, 20 μM had an inhibitory effect. Importantly, the formation of resveratrol-induced networks was completely reversed by the addition of VEGF-neutralizing antibody, confirming that the angiogenic effect of resveratrol was VEGF-dependent. These findings were confirmed using primary endothelial cells isolated from human umbilical veins (Fig. 2B).

We further used chick chorioallantoic membrane assay to test the angiogenic effect of resveratrol *in vivo*. Consistent with the *in*

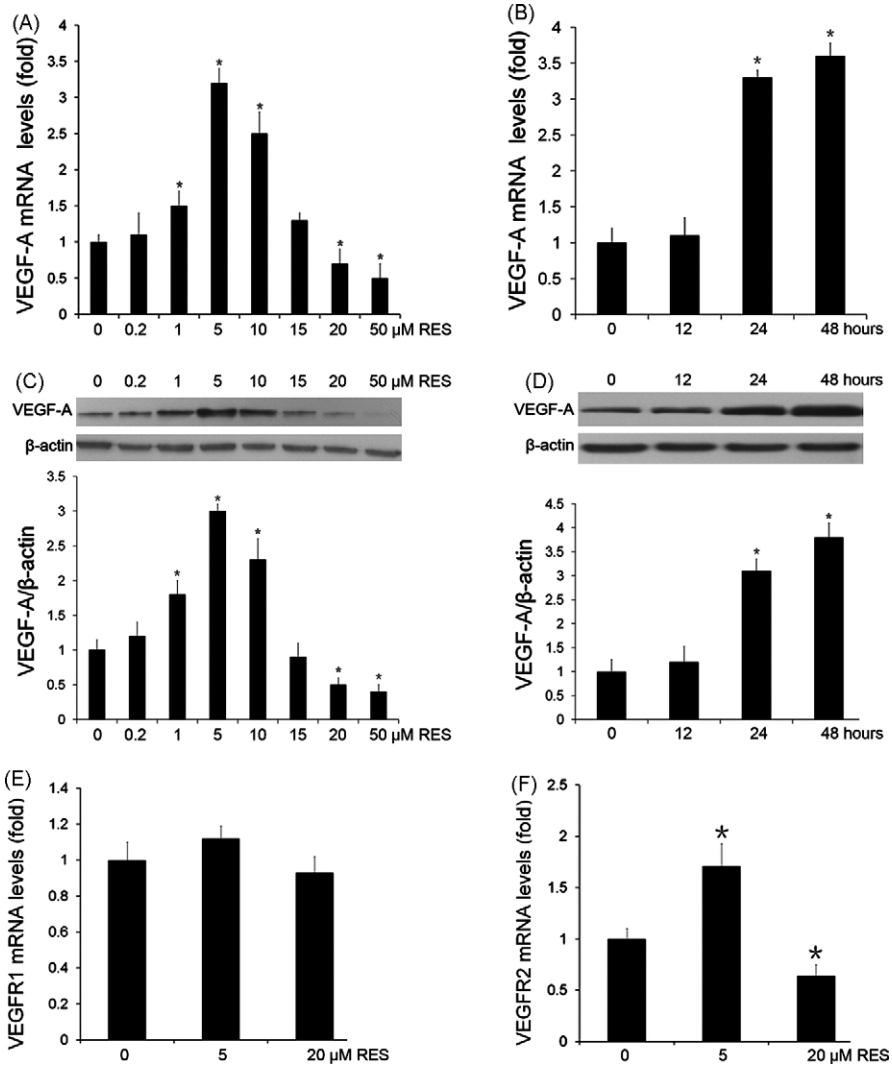

Fig. 1. Resveratrol (RES) regulates *VEGF* and *VEGFR2* expression in a biphasic manner. (A and B) Expression of *VEGF* at mRNA level. (A) HUVECs were treated with increasing concentration of RES for 24 h. (B) HUVECs were treated with 5 μM RES for different time intervals. Total RNAs were prepared, and *VEGF* mRNA levels were determined by Real-time PCR using *VEGF-A* specific primers. (C and D) Levels of VEGF determined by Western blot. (C) HUVECs were treated with increasing concentration of RES for 24 h. (D) HUVECs were treated with 5 μM RES for different time intervals. Cell lysates were subjected to immunoblotting with antibody against VEGF, and β-actin was used as the internal control. Signal intensities were determined by densitometry. The expression level of VEGF in control was arbitrarily set to 1, and the relative expression level of RES-treated cells was calculated accordingly. (E) Expression of *VEGFR1* at mRNA level. (F) Expression of *VEGFR2* at mRNA level. The expression level in control was arbitrarily set to 1, and the relative expression level of RES-treated cells was calculated accordingly. Each bar represents mean ± SEM of three independent experiments performed in triplicate. $^{*}P < 0.05$ for RES-treated cells versus control cells treated with DMSO.

vitro data, while 5 μM resveratrol significantly promoted the formation of microvessel structures, 20 μM resveratrol or a combination of 5 μM resveratrol and VEGF-neutralizing antibody led to a significant reduction in angiogenesis (Fig. 2C).

3.3. Resveratrol-induced up-regulation of VEGF involves nuclear accumulation of β-catenin

VEGF transcription was found to be elevated in response to stabilization of β-catenin in several in vitro systems, including vascular endothelial cells [5,13,14,28]. To uncover the molecular mechanisms underlying resveratrol-induced up-regulation of *VEGF*, we first examined the amount of β-catenin and its subcellular distribution in resveratrol-treated HUVECs by Western blot analysis of fractioned cytosolic and nuclear extracts. LiCl, which regulates gene expression by activating the Wnt signaling pathway [29], was used as a positive control (at 10 mM). Although a change in total β-catenin level was not detected, there was a significant increase in the nuclei, at the expense of cytosolic β-catenin, after incubation in 5 μM resveratrol or 10 mM LiCl for 24 h (Fig. 3A and B). To verify these results, we used immunofluorescence staining to visualize the subcellular localization of β-catenin. As shown in Fig. 3C, β-catenin was mainly detected in the nuclei after treatment with 5 μM resveratrol for 24 h, confirming the results by Western blot analysis.

3.4. Resveratrol stimulates VEGF expression in a β-catenin/TCF-dependent manner

To prove that resveratrol-induced accumulation of β-catenin is responsible for the elevation of *VEGF* expression, we assessed the effects of β-catenin knockdown via RNA interference on VEGF expression. As shown in Fig. 4A, treatment of HUVECs with siRNA targeting β-catenin, but not with nonspecific siRNA, significantly reduced the production of β-catenin protein. In response to the decrease in the production of β-catenin, the resveratrol-induced *VEGF* expression was significantly decreased (Fig. 4B), indicating that the increased production of VEGF stimulated by resveratrol was β-catenin-dependent.

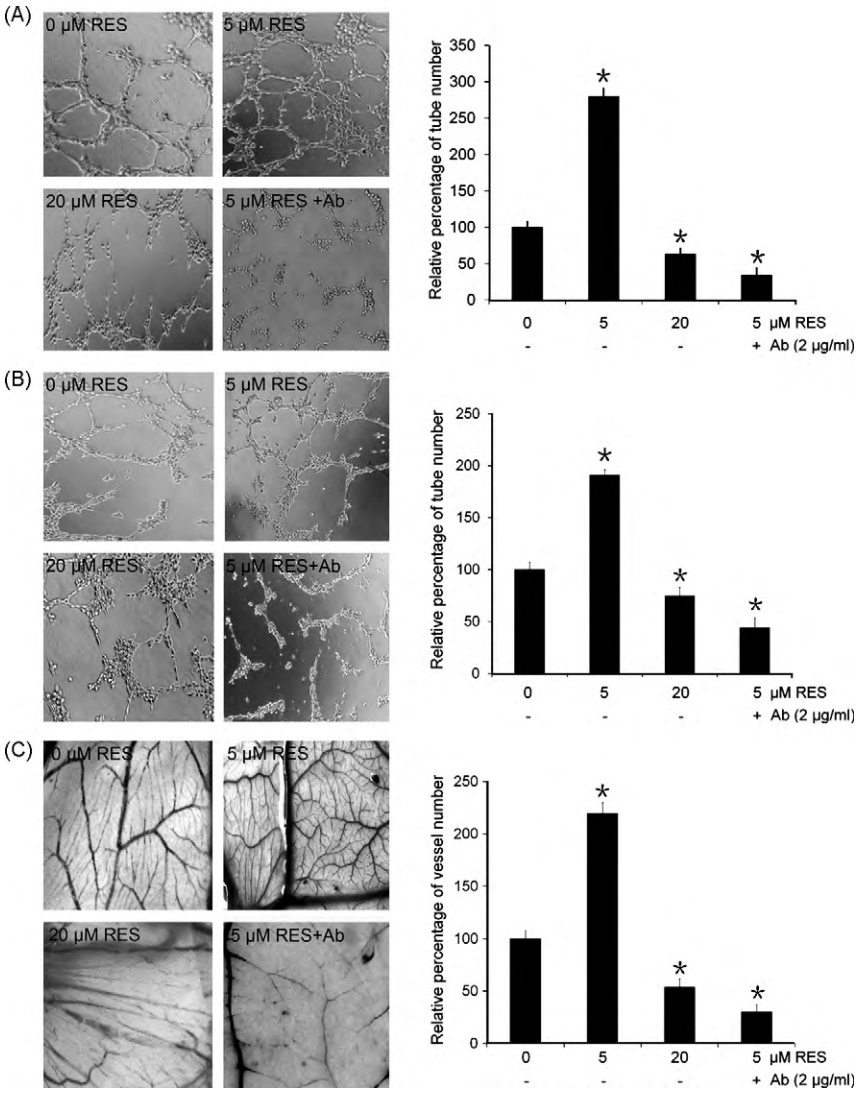

Fig. 2. The effect of RES on angiogenesis in HUVECs and in CAMs. HUVECs or primary endothelial cells were seeded on Matrigel-coated 48-well plates. Cells were treated without or with RES or with a combination of RES and VEGF-Ab. (A) Effect of resveratrol on the formation of tube-like structures in HUVECs (cell line). (B) Effect of resveratrol on the formation of tube-like structures in primary human endothelial cells. (C) Effect of resveratrol on the branching blood vessels in CAMs. Each experiment was repeated at least three times. The panels on the right showed the mean ± SEM of the number of tube-like structure relative to the control, which was set to be 100%. $^{*}P < 0.05$ for RES-treated cells versus control cells treated with DMSO.

In the nucleus, β-catenin forms complexes with members of the TCF/LEF family of transcription factors to activate the expression of target genes. The resveratrol-induced increase in nuclear β-catenin could lead to the activation of β-catenin/TCF/LEF-mediated genes. Therefore we examined the effect of resveratrol on the transcriptional activity of β-catenin/TCF using a reporter assay. The luciferase reporters, which contain the open reading frame of the luciferase gene, a minimal thymidine kinase (TK) promoter and either wild type (TOPFLASH) or mutated (FOPFLASH) binding sites for the β-catenin/TCF complex, were transiently transfected into HUVECs, along with pRL-TK reporter vector to normalize for transfection efficiency. Six h after transfection, the cells were treated with 5 μM resveratrol or 10 mM LiCl for 24 h, and luciferase activity was determined. As shown in Fig. 4C, treatment with 5 μM resveratrol or LiCl increased TOPFLASH activity by 2-fold over the solvent control. These results demonstrate that 5 μM of resveratrol may activate the transcription of genes targeted by TCF transcription factor.

The promoter of *VEGF-A* gene contains a putative TCF site, CAAAG, located at −345 to −349 (Fig. 4D). Wnt/β-catenin may directly stimulate *VEGF-A* gene expression by acting on this TCF-binding site. To further confirm that resveratrol up-regulates *VEGF* expression through Wnt/β-catenin signaling pathway, we tested the role of the TCF-binding site in the promoter of *VEGF* in response to resveratrol treatment. We constructed luciferase reporters that contain wild-type, CAAAG, and mutated TCF element, CGAGG, respectively, in their promoters. Transient transfection experiments showed that resveratrol increased the luciferase activity only in construct containing wild-type TCF, but not in that containing mutated TCF binding site (Fig. 4E). These results further demonstrate that resveratrol exerts its effects in a β-catenin/TCF-dependent manner.

3.5. Resveratrol treatment induces phosphorylation and inactivation of GSK3β

In the absence of the Wnt signaling pathway, β-catenin in cytosolic pool is phosphorylated by GSK3β and degraded by ubiquitin-mediated proteosome. GSK3β itself can become inactivated by its phosphorylation, resulting in the stabilization of

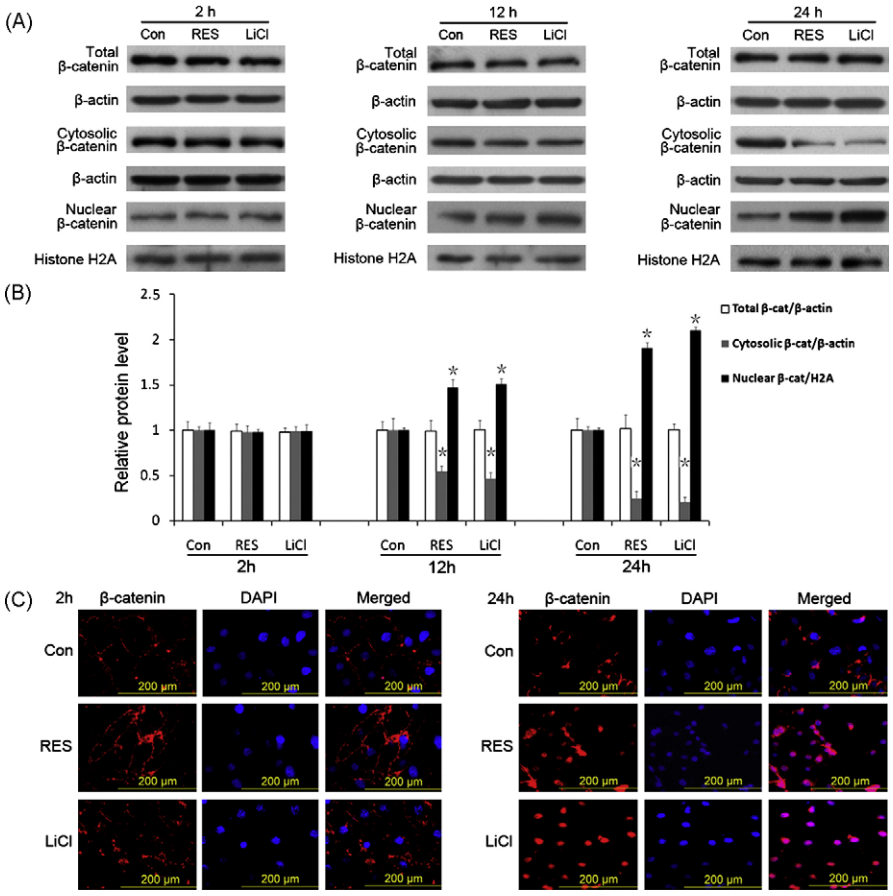

Fig. 3. RES treatment resulted in nuclear accumulation of β-catenin. (A) Representative Western blot results of β-catenin Total, cytosolic and nuclear fractions of protein lysates were prepared from HUVECs treated with DMSO (Con), 5 μM RES and 10 mM LiCl for indicated hours. Western blot was probed with antibodies against β-catenin. Equal loading was confirmed by the presence of β-actin and histone 2A, respectively. (B) Quantitative summary of the intensities of Western blot bands. The protein level in control was arbitrarily set to 1, and the relative protein level of RES-treated cells was calculated accordingly. (C) Nuclear accumulation of β-catenin shown by immunofluorescence. Anti-β-catenin antibody and DAPI showed relative nuclear and cytoplasmic distribution of β-catenin in the cells treated with DMSO (Con), 5 μM RES and 10 mM LiCl for 2 h and 24 h. Each experiment was repeated three times.

β-catenin protein and subsequently an increased transcription of its target genes [10]. We therefore examined the status of GSK3β phosphorylation in resveratrol-treated HUVEC cells. As shown in Fig. 5A, the levels of GSK3β phosphorylated at Ser9 were significantly increased after cells were treated with 5 μM resveratrol or 10 mM LiCl. In contrast, incubation in 20 μM resveratrol resulted in a decrease in the levels of phosphorylated GSK3β, which was accompanied by a significant increase in the amount of phosphorylated β-catenin and a decrease in the nuclear accumulation of β-catenin (Fig. 5B). It should be noted that the total GSK3β protein levels in HUVECs remain unchanged at low or high concentrations of resveratrol. These results indicate that the change in the level of nuclear β-catenin in response to resveratrol is only related to the kinase activity of GSK3β.

3.6. PI3K/AKT and MEK/ERK pathways are involved in resveratrol-induced inactivation of GSK3β

Recent studies suggest that resveratrol can activate the PI3K and the MAPK pathways [17,23], which are known to be critical for angiogenesis. Since GSK3β, a downstream target of PI3K/AKT and MEK/ERK signaling pathways, can be inactivated by phosphorylation at its Ser9 residue, we evaluated whether PI3K/Akt and MEK/ERK signaling pathways were involved in the phosphorylation of GSK3β in response to resveratrol treatment. Western blot analysis with antibodies against the active forms of AKT and ERK showed that 5 μM resveratrol greatly increased the levels of pAkt and pERK, which peaked at 6 h and then return to baseline (Fig.6A and B), while the total amount of Akt and ERK remained unchanged. As expected, the phosphorylation level of GSK3β was significantly increased and peaked at 24 h. To further evaluate the role of PI3K/Akt and MEK/ERK signaling pathways in resveratrol-induced GSK3β inactivation, we pretreated cells with the PI3K inhibitor LY294002 and the MEK inhibitor PD98059, respectively. Both treatments significantly decreased the resveratrol-induced activation of Akt and ERK, and blocked resveratrol-induced phosphorylation of GSK3β (Fig. 6A and B). In contrast, 20 μM resveratrol significantly decreased the levels of pAkt and pERK, with a noticeable drop at 6 h (Fig. 6C and D). Accordingly, the phosphorylated form of GSK3β dropped to the lowest level at 24 h. Combination of 20 μM resveratrol with LY294002 or PD98059 further decreased the phosphorylation of GSK3β (Fig. 6C and D).

We further determined whether the inhibition of PI3K/Akt and MEK/ERK signaling pathways affected resveratrol-induced network formation in HUVECs and in CAMs. As shown in Fig. 7, both LY294002 and PD98059 were able to inhibit the formation of resveratrol-induced networks, indicating that the activation of PI3K/Akt and MEK/ERK pathways is required for capillary tube formation. Collectively, our data indicate that low concentration of resveratrol can activate PI3K/Akt and MEK/ERK signaling pathways in HUVECs, leading to the inactivation of GSK3β and increased

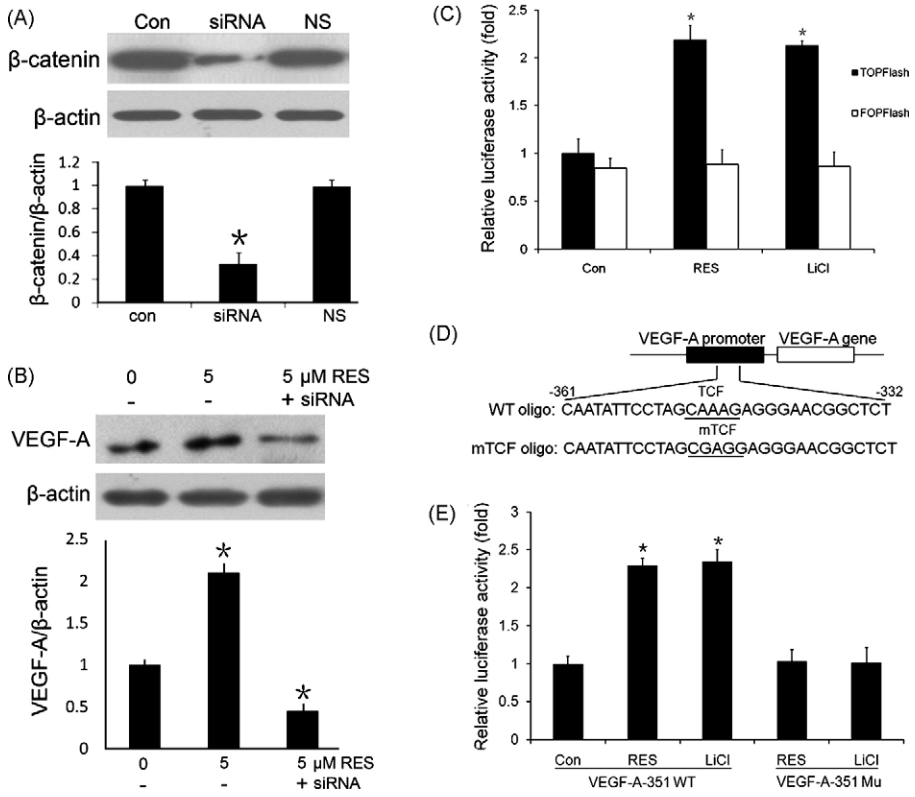

Fig. 4. RES stimulates *VEGF* expression in a β-catenin/TCF-dependent manner. (A) Representative Western blot results of β-catenin. Cell lysates of HUVECs transfected with β-catenin-siRNA or nonspecific siRNA (NS) were blotted with anti-β-catenin and β-actin antibodies. (B) HUVECs were transfected with β-catenin-siRNA or nonspecific siRNA (NS) and post-treated with 5 μM RES for 24 h. Immunoblot analysis was performed with anti-VEGF and anti-β-actin antibodies. Signal intensities were determined by densitometry. The expression level in control (Con) was arbitrarily set to 1, and the relative expression level of treated cells was calculated accordingly. (C) TCF-dependent transcription. HUVECs were transiently transfected with TOPFLASH or FOPFLASH along with pRL-TK vector. Six hours after transfection, cells were treated with DMSO (Con), 5 μM RES or 10 mM LiCl for 24 h, and the relative luciferase activity was determined. The activity of TOPFLASH construct in control cells was set to 100%, and the relative luciferase activity of the others was calculated accordingly. Each bar represents the value of mean ± SEM. $^{*}P < 0.05$. (D) Schematic illustration of the putative TCF response elements in the promoter of *VEGF-A* gene. The sequences of the region containing TCF-binding sites were shown. Two point mutations were introduced to change the core sequence from CAAAG to CGAGG. (E) RES activated *VEGF* promoter activity. Luciferase constructs containing wild type or mutant TCF-binding site were transiently transfected into HUVECs along with pRL-TK vector. Six hours after transfection, cells were treated with DMSO (Con), 5 μM RES or 10 mM LiCl for 24 h. The ratio of the relative luciferase activity between control and RES or LiCl was calculated. Each bar represents the value of mean ± SEM. $^{*}P < 0.05$. Each experiment was performed in triplicate.

nuclear accumulation of β-catenin, which in turn promotes *VEGF* expression and the subsequent capillary tube formation. In contrast, high concentration of resveratrol can reduce the activity of PI3K/Akt and MEK/ERK signaling pathways and eventually inhibit angiogenesis.

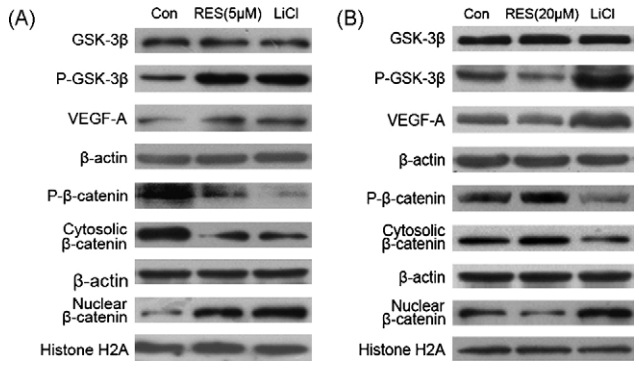

Fig. 5. Involvement of GSK3β in RES-induced β-catenin stabilization and *VEGF* expression. Total, cytosolic, and nuclear proteins were prepared from the HUVECs treated with DMSO (Con), 5 μM (panel A) and 20 μM (panel B) RES, and 10 mM LiCl for 24 h. Western blots were probed with antibodies against GSK3β, phosphorylated GSK3β (P-GSK3β)(Ser9), VEGF-A, phosphorylated β-catenin (P-β-catenin), β-catenin. β-actin and histone 2A were used as loading control. Each experiment was performed three times.

4. Discussion

Resveratrol has been shown to have cardioprotective effect [16–18]. On the other hand, many studies also showed that it possesses antiangiogenic effect [30–37]. The mechanisms by which resveratrol regulates cardiovascular function were also shown to be multifaceted [17–23]. In the present study we found that resveratrol had opposite effects on the expression of *VEGF* and angiogenesis depending on the concentration at which it was applied to human endothelial cells. While low concentrations of resveratrol can up-regulate the expression of *VEGF* and promote angiogenesis, high concentrations of resveratrol inhibited angiogenesis formed by HUVECs. Importantly, we showed for the first time that the regulation of *VEGF* expression and angiogenesis by resveratrol can be mediated by GSK3β/β-catenin pathway. We also demonstrated that the PI3K/Akt and MEK/ERK signaling pathways act upstream of GSK3β and contribute to the inactivation of GSK3β.

The expression of *VEGF* is differentially regulated in various physiological and pathological conditions. For example, HIF is known to up-regulate VEGF in promoting tumor angiogenesis [30]. Resveratrol has been shown to lower the levels of intracellular HIF-1 and VEGF in ovarian cancer cells [31], human papillomavirus-transfected cervical cancer cells [32], human tongue squmous carcinoma cells and hepatoma cells [33], and endometrial cancer cells [34]. It has also been shown to suppress the growth of new blood vessels in animals. Oral administration of resveratrol

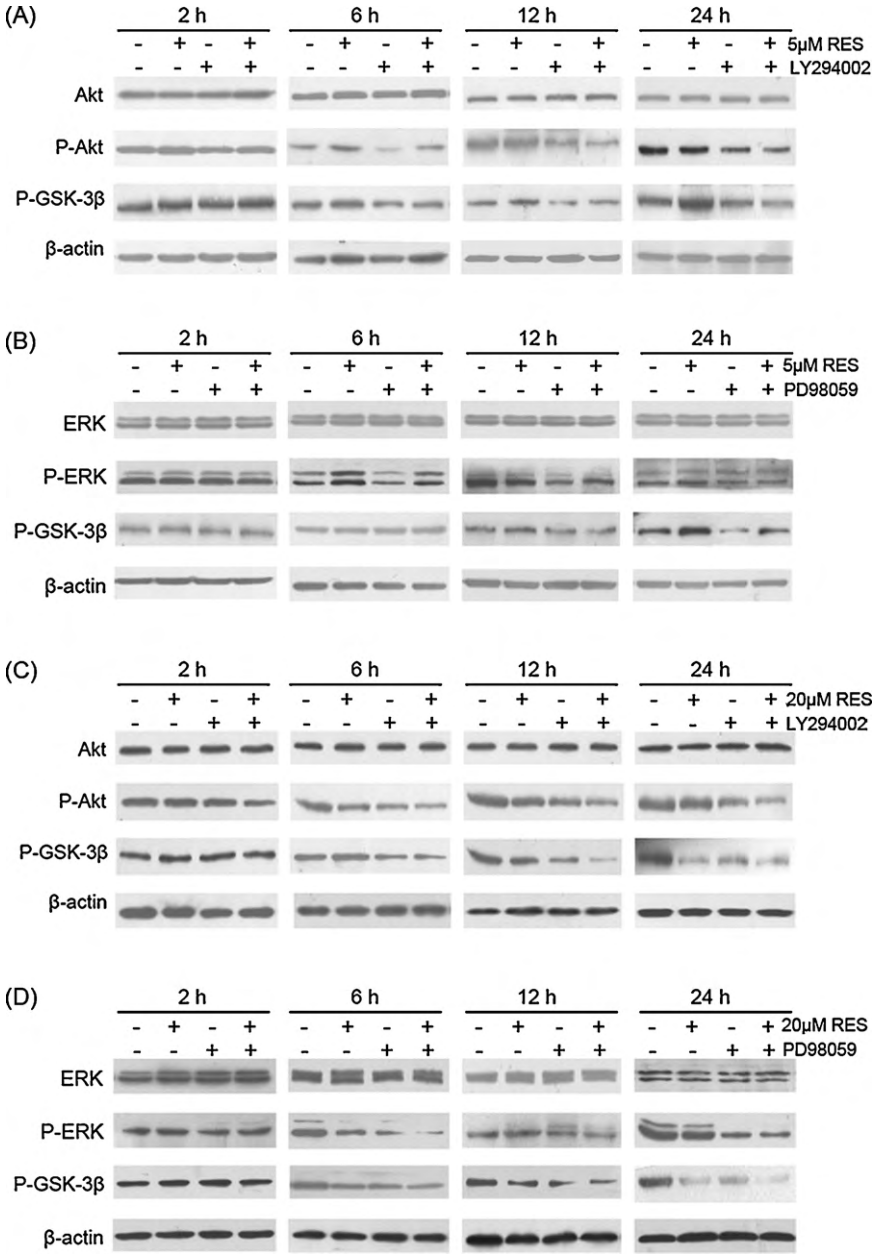

Fig. 6. RES activates PI3K/Akt and MEK/ERK signaling pathways. (A) HUVECs were treated with RES (5 μM) and the PI3K inhibitor LY294002 (LY). (B) HUVECs were treated with RES (5 μM) and MEK inhibitor PD98059 (PD). (C) HUVECs were treated with RES (20 μM) and the PI3K inhibitor LY294002 (LY). (D) HUVECs were treated with RES (20 μM) and MEK inhibitor PD98059 (PD). The cell lysates were prepared, and total Akt, ERK, and their phosphorylated forms (P-Akt and P-ERK), and phosphorylated GSK3β (Ser9) (P-GSK3β) were determined with the corresponding antibodies.

significantly inhibits the growth of murine fibrosarcomas and delays angiogenesis-dependent wound healing in mice [35]. However, we demonstrated in this study that the expression of *VEGF* in HUVECs is differentially regulated depending on the concentration of resveratrol. Resveratrol at low concentrations increased the expression of *VEGF*, whereas at high concentrations it had an inhibitory effect. These results suggest that resveratrol may act either as a promoter or as an inhibitor of *VEGF* expression, depending on the cell type it is applied to and the concentration it is applied at. It appears that the effect of resveratrol on angiogenesis also varies in different experimental settings. For example, whereas addition of exogenous VEGF can promote angiogenesis in HUVECs, this VEGF-induced angiogenesis can be inhibited by resveratrol even at very low concentrations [36]. However, as shown in our study, it is clear that resveratrol at low concentrations possesses angiogenic activity via the up-regulation of the expression of endogenous VEGF in the absence of exogenous VEGF. A study using zebrafish as a model system also indicated that while having angiogenic effects at low concentrations, resveratrol and its derivatives were able to inhibit angiogenesis at high concentrations [37].

Unlike the regulation of *VEGF* expression by HIF in cancer cells, change in expression of *VEGF* in HUVECs in response to resveratrol is mediated via GSK3β/β-catenin/TCF signaling pathway. Elevated β-catenin signaling in endothelial cells was shown to promote angiogenesis [14]. Interestingly, resveratrol is also capable of promoting osteoblastic differentiation by up regulating β-catenin [24]. Treatment of mesenchymal stem cells by resveratrol resulted in the phosphorylation and inactivation of GSK3β, which in turn led to the nuclear accumulation of β-catenin, transcriptional up-regulation of β-catenin/TCF target genes required for bone formation.

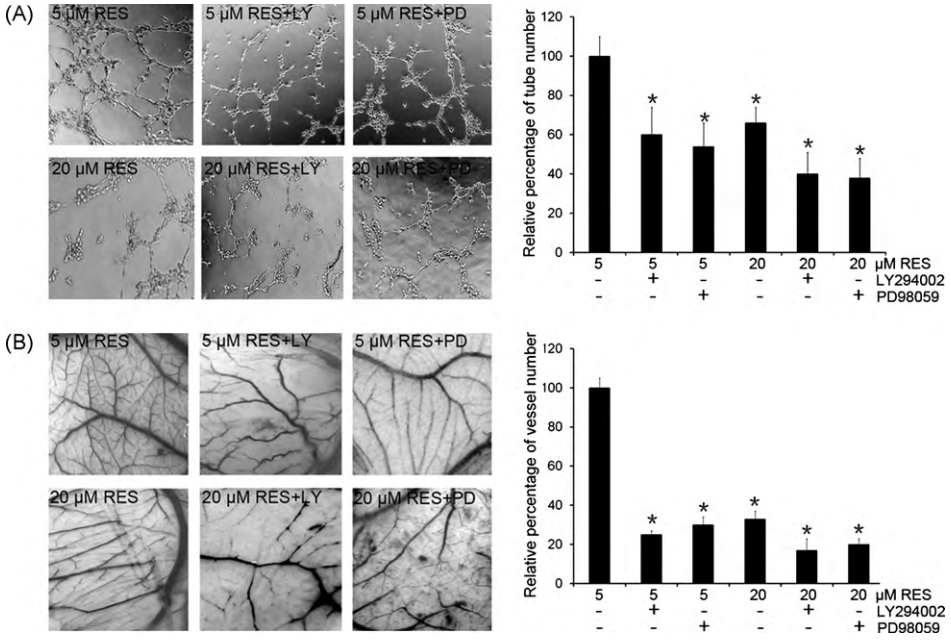

Fig. 7. RES-induced angiogenesis was blocked by the PI3K inhibitor and MEK inhibitor. (A) HUVECs were seeded on growth factor-reduced Matrigel-coated plate. Cells were treated with RES (5 μM or 20 μM) in the absence or presence of LY294002 (LY) or PD98059 (PD). The number of tube-like structures was scored. $^{*}P < 0.05$ when compared to 5 μM RES treatment. (B) RES-induced angiogenesis was blocked by the PI3K inhibitor and MEK inhibitor in vivo by CAM assay. Cells were treated with RES (5 μM or 20 μM) in the absence or presence of LY294002 (LY) or PD98059 (PD). The number of branching vessels was scored. Each experiment was repeated three times. $^{*}P < 0.05$ when compared to 5 μM RES treatment.

We showed that inactivation of GSK3β in response to resveratrol treatment involves both PI3K/Akt and MEK/ERK signaling pathways. These data indicate that PI3K/Akt and MEK/ERK pathways probably converge at β-catenin signaling pathway, via the GSK3β nodal point. Many studies showed that the angiogenic effect of VEGF is mediated by PI3K/Akt pathway [38–41]. Moreover, the induction of VEGF by HIF also depends on PI3K/Akt [41,42]. We showed here that the induction of angiogenesis by resveratrol can be inhibited by PI3K inhibitor LY294002. Therefore, while Akt serves to enact the function of VEGF, it is also required in the production of VEGF. Activation of Akt may represent a key step in the positive feedback loop that amplifies the angiogenic effect of VEGF in endothelial cells.

By acting on PI3K/Akt and MEK/ERK en route to inactivate GSK3β, resveratrol exerts its biological effects by skipping the early steps of the canonical Wnt signaling pathway. Consistent with the results in this study, the involvement of PI3K/Akt and/or MEK/ERK in the regulation of the β-catenin signaling pathway has been described in other cell systems [43–45]. While the mechanisms by which resveratrol activates PI3K/Akt and MEK/ERK remain unclear, the knowledge of regulation of GSK3β by resveratrol through modulating MEK/ERK and PI3K/Akt pathways may have physiological significance in management of diabetic retinopathy, rheumatoid arthritis, psoriasis, cardiovascular diseases, and cancer.

In summary, our study showed that resveratrol may have opposite effects on angiogenesis depending on the concentration it is applied. This effect of resveratrol is executed through the nodal point of GSK3β, the negative regulator of β-catenin/TCF pathway. Low dose of resveratrol can up-regulate β-catenin signaling pathway that culminates in the production of VEGF. These results shed light on the mechanism of action of resveratrol and also provide more insights into the possible therapeutic use of resveratrol for treating diseases resulting from insufficient or excessive blood vessel formation.

Acknowledgements

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