# Magnolol inhibits angiogenesis by regulating ROS-mediated apoptosis and the PI3K/AKT/mTOR signaling pathway in mES/EB-derived endothelial-like cells

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**Abstract.** Magnolol, a neolignan from the traditional medicinal plant *Magnolia obovata,* has been shown to possess neuroprotective, anti-inflammatory, anticancer and anti-angiogenic activities. However, the precise mechanism of the anti-angiogenic activity of magnolol remains to be elucidated. In the present study, the anti-angiogenic effect of magnolol was evaluated in mouse embryonic stem (mES)/embryoid body (EB)-derived endothelial-like cells. The endothelial-like cells were obtained by differentiation from mES/EB cells. Magnolol (20 $\mu$M) significantly suppressed the transcriptional and translational expression of platelet endothelial cell adhesion molecule (PECAM), an endothelial biomarker, in mES/EB-derived endothelial-like cells. To further understand the molecular mechanism of the suppression of PECAM expression, signaling pathways were analyzed in the mES/EB-derived endothelial-like cells. Magnolol induced the generation of reactive oxygen species (ROS) by mitochondria, a process that was associated with the induction of apoptosis as determined by positive Annexin V staining and the activation of cleaved caspase-3. The involvement of ROS generation by magnolol was confirmed by treatment with an antioxidant, N-acetyl-cysteine (NAC). NAC inhibited the magnolol-mediated induction of ROS generation and suppression of PECAM expression. In addition, magnolol suppressed the activation of MAPKs (ERK, JNK and p38) and the PI3K/AKT/mTOR signaling pathway in mES/EB-derived endothelial-like cells. Taken together, these findings demonstrate for the first time that the anti-angiogenic activity of magnolol may be associated with ROS-mediated apoptosis and the suppression of the PI3K/AKT/mTOR signaling pathway in mES/EB-derived endothelial-like cells.

## Introduction

Angiogenesis, the formation of new microvessels from the pre-existing blood vasculature, is a highly regulated process that involves the activation, growth and migration of endothelial cells and capillary morphogenesis (1,2). Although angiogenesis is an essential process in embryonic vascular development, organ regeneration and wound healing, a variety of pathological diseases, including cancer, rheumatoid arthritis and diabetic retinopathy, also involve angiogenic events (1,3). Therefore, the identification of anti-angiogenic agents with new mechanisms of action would be an attractive strategy for studying angiogenic processes and providing potential lead candidates for the development of new drugs associated with angiogenesis (4). There is increasing evidence that reactive oxygen species (ROS) are involved in the regulation of angiogenesis. Although high levels of ROS may result in oxidative damage to various cellular components and, finally, in cell apoptosis (5), low levels of ROS have been demonstrated to be involved in signal transduction cascades that regulate endothelial cell growth, migration and organization into tubular network structures, which are critical steps in angiogenesis (6,7). The ROS-induced apoptotic pathway is also considered the intrinsic or mitochondrial pathway, in which intrinsic death stimuli directly or indirectly activate the mitochondrial pathway (8). The mitogen-activated protein kinase (MAPK) and AKT/mTOR signaling pathways play important roles in the regulation of many cellular processes, including cell growth and proliferation, differentiation and apoptosis (9). Previous studies have also indicated that ROS can induce or mediate the activation of MAPK signaling pathways (10) and MAPK signaling proteins are involved in growth arrest and apoptosis via ROS generation (11).

Embryonic stem (ES) cells are pluripotent cells established from the inner cell mass of blastocysts and have the potential to differentiate into many cell types, such as hematopoietic cells, neuronal cells, cardiomyocytes, muscle cells, epithelial cells and endothelial cells (12,13). Therefore, ES cells are considered a useful tool for the study of angiogenesis,

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including the processes of angioblast differentiation, proliferation, migration, endothelial cell-cell adhesion and vascular morphogenesis (12,14,15). One promising approach for differentiating ES cells into endothelial cells is the use of embryoid bodies (EBs), which are embryo-like aggregates of ES cells. Therefore, the ES/EB system represents a good *in vitro* model for the study of vasculogenesis as well as angiogenesis (16). In addition, ES-derived EB cells closely resemble their *in vivo* counterparts and thus provide a useful *in vitro* model for the study of specific cell signaling systems (17,18). Recently, we also demonstrated the usefulness of mES-derived endothelial cell systems in the evaluation of the effect of 5-FU on vasculogenesis and the anti-angiogenic effects of natural product-derived compounds (19,20).

Magnolol is a neolignan from the bark of *Magnolia obovata* Thunberg (Magnoliaceae), which has traditionally been used to treat gastrointestinal tract disorders in Asian countries (21,22). A variety of pharmacological activities of magnolol have been reported, including anti-inflammatory (23), anti-oxidant (24), anticancer and anti-angiogenic effects. The anticancer activity of magnolol was found to be able to suppress the proliferation of cancer cells by inhibiting DNA synthesis and inducing apoptosis (25-27). However, the precise mechanism of action for the anti-angiogenic activity of magnolol remains to be elucidated.

In the present study, we demonstrated that magnolol might be able to suppress angiogenesis through the inhibition of ROS-mediated MAPK and AKT/mTOR signaling in mES/EB-derived endothelial-like cells.

## Materials and methods

*Reagents and antibodies*. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), gelatin and HRP-conjugated anti-mouse and anti-rabbit antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Collagen type I was purchased from BD Biosciences (San Jose, CA, USA). Phospho-specific anti-p38(Thr[180]/Tyr[182]), anti-JNK(Thr[183]/Tyr[185]), anti-PI3K, anti-PDK1, anti-AKT(Ser[473]), anti-mTOR(Ser[2448]), anti-p70S6K(Thr[389]), anti-4EBP1(Thr[37]/Thr[46]) and procaspase-3 antibodies, the MAPK inhibitors; SB203580, PD98059 and SP600125; and the PI3K inhibitor LY294002 were purchased from Cell Signaling Technology (Danvers, MA, USA). The HRP-conjugated β-actin, phospho-ERK(Thr[202]/Tyr[204]), Bcl-xL, Bax and PECAM antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 2'7'-Dichlorofluorescein diacetate (H₂DCFDA) and Alexa Fluor 594-labeled chicken anti-rat IgG were purchased from Molecular Probes (Invitrogen, Carlsbad, CA, USA). Magnolol (purity >98%, Fig. 1A) isolated from the bark of *Magnolia obovata* was provided by Dr K. Bae (28). The compound was dissolved in 100% DMSO. A 100-mM stock solution of magnolol was prepared and stored at -20°C until use.

*Cell culture*. Mouse D₃ ES cells (ATCC cat. no. CRL-1934, Rockville, MD, USA) were co-cultured with mitomycin C-treated mouse embryonic fibroblasts in high-glucose DMEM (Invitrogen) containing 10% fetal bovine serum (Hyclone, Ogden, UT, USA), 1,000 U/ml leukemia inhibitory factor (LIF) (Chemicon, Temecula, CA, USA) and basic ES medium components [50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen), 1% non-essential amino acids (Invitrogen) and 0.1 mM β-mercaptoethanol (Invitrogen)]. The hanging drop method (20 μl per drop; 1x10⁵ cells/ml) was used to induce differentiation as previously described (20,29). The EBs were formed by incubating the hanging drop cultures for 3 days. The resulting EBs were transferred onto gelatin-coated chamber slides (Nunc, Denmark) or 60-mm dishes to allow attachment. Endothelial cell differentiation was induced in EBs by switching the culture conditions to medium containing EBM-2, 5% FBS, a growth factor cocktail and ascorbic acid (EGM2-MV Bullet kit; Lonza, Walkersville, MD, USA). The cell culture and endothelial differentiation conditions for the mES cells followed the protocol in our previous report (20,30).

*Growth inhibition assay*. The growth inhibition activity in cultured mES-derived endothelial-like cells was determined using MTT assays as previously described (20,30). Briefly, cells were seeded at a density of 5,000 cells/well into 96-well plates. On differentiation day 10, the cells were exposed to various concentrations of magnolol for 24 h. After incubation, MTT solution was added and the cells were incubated for an additional 4 h. The resulting formazan was dissolved in DMSO and the absorbance was detected at 570 nm with a VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA). After treatment of cells with magnolol for 24 h at differentiation day 10, the cytotoxicity of magnolol was tested using the CytoTox 96® Non-Radioactive Cytotoxicity assay (Promega, Madison, WI, USA). The effects on cell viability and the cytotoxicity activity of magnolol were calculated as percentages relative to the solvent-treated control.

*Immunocytochemistry*. Cells were plated onto confocal dishes and induced to differentiate into endothelial-like cells by incubation in EGM-2 medium for 10 days. The mES/EB-derived endothelial-like cells were incubated with magnolol with or without 5 mM NAC for 24 h. After incubation, the cells were fixed with 4% paraformaldehyde. The cells were blocked with blocking solution containing 1% BSA/PBS for 30 min and then incubated with rat anti-mouse PECAM (1:200) (Santa Cruz) overnight at 4°C. After being washed, the cells were incubated with Alexa Fluor 594-labeled chicken anti-rat IgG (1:1,000) (Invitrogen). After staining, the cover slips were mounted with medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). Confocal microscopy was performed using a Zeiss Model 710 (Carl Zeiss, Jena, Germany).

*RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)*. Cells were dissolved using TRIzol® (Invitrogen) and total RNA was extracted according to the manufacturer's protocol. The total cellular RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Reverse transcription was performed using 2 μg of purified total RNA and the SuperScript First-Strand Synthesis System (Invitrogen). The synthesized cDNAs were amplified by PCR. The primers used for RT-PCR are listed in Table I. The thermal cycling parameters were as follows: 5 min at 94°C, 30 amplification
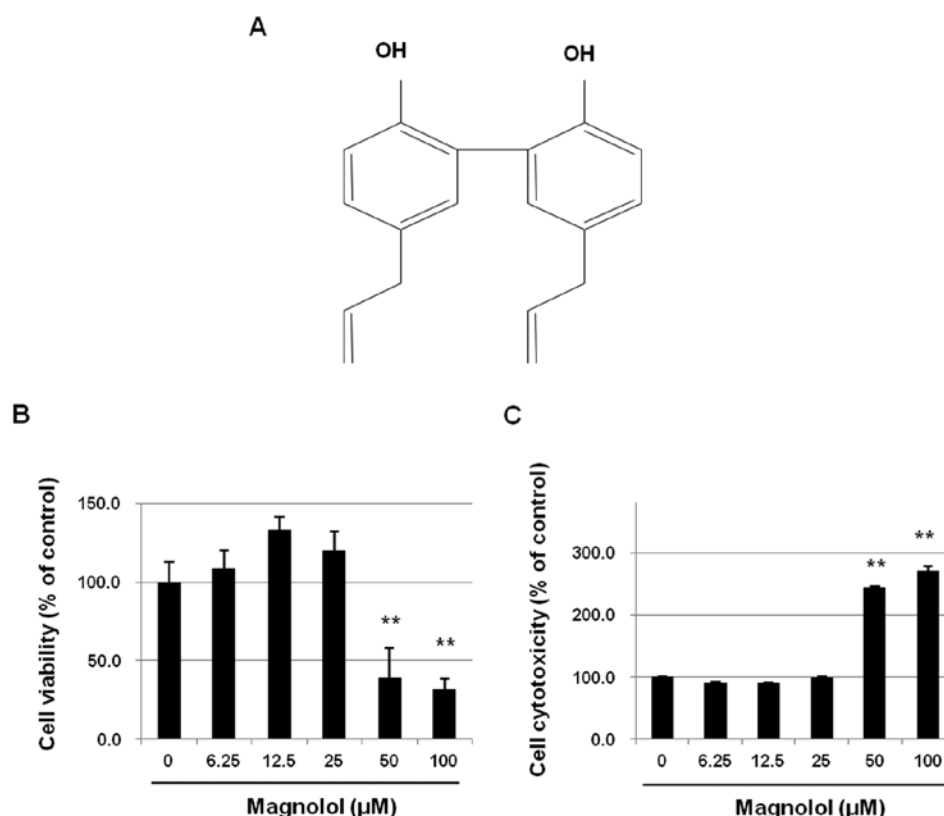
Figure 1. Effect of magnolol on cell viability and the cytotoxicity of magnolol in mES/EB-derived endothelial-like cells. (A) Chemical structure of magnolol. (B) On endothelial differentiation day 10, mES/EB-derived endothelial-like cells were exposed to magnolol (0-100 $\mu$M) for 24 h and then the cell viability was measured by the MTT assay. (C) LDH leakage was evaluated after 24 h of treatment with magnolol (0-100 $\mu$M) using an LDH assay kit. Data are presented as mean ± SD in triplicate tests. [**]$P<0.01$ compared with control.

Table I. Sequences of the primer pairs of specific target genes used in RT-PCR.

| Gene | Primer sequences | Product size (bp) |
|------|------------------|-------------------|
| PECAM | F 5'-CCATCATGGGAGGTGATGAA-3' | |
| | R 5'-GATACGCCATGCACCTTCAC-3' | 278 |
| GAPDH | F 5'-GGAGCCAAAAGGGTCATCAT-3' | |
| | R 5'-GTGATGGCATGGACTGTGGT-3' | 212 |

cycles (denaturation at 94°C for 30 sec, annealing at 55-60°C for 30 sec and extension at 72°C for 30 sec) and a final extension at 72°C for 5 min. The amplified products were separated on 1.5% agarose gels. The gels were stained with SYBR® Gold staining solution (Invitrogen) and visualized by UV transillumination (GelDoc™ XR, BioRad Molecular Imager).

*Flow cytometric analysis of apoptosis.* To determine the level of apoptosis following magnolol exposure for 24 h during the differentiation of mES cells into endothelial cells, the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Pharmingen) was used. In this assay, Annexin V-FITC binds to phosphatidylserine, which translocates to the outer leaflet of the plasma membrane during the early stages of cell apoptosis. Therefore, the apoptotic cells were specifically stained with Annexin V-FITC, whereas the necrotic cells were doubly stained with both Annexin V-FITC and PI. The cells were suspended in binding buffer at a final cell concentration of $1 \times 10^5$ cells/ml and incubated with both Annexin V-FITC and PI for 25 min in the dark. The DNA contents of the stained cells were analyzed using CellQuest Software and a FACS Vantage SE flow cytometer (Becton-Dickinson, Germany).

*Quantification of reactive oxygen species production.* The intracellular ROS levels were measured using the fluorescent dye $H_2$DCFDA (Molecular Probes). Cells were induced to differentiate into endothelial-like cells by incubation in EGM-2 medium for 10 days. The mES/EB-derived endothelial-like cells were incubated with magnolol for 24 h. Then, the cells were washed twice, stained with 20 $\mu$M $H_2$DCFDA (Molecular Probes) for 30 min and washed twice. The resulting compound, 2'7'-dichlorofluorescein diacetate ($H_2$DCFDA), reacts with ROS to form a fluorescent compound, dichlorofluorescin (DCF). The amount of intracellular DCF was measured using flow cytometer (Becton-Dickinson).

*Measurement of the mitochondrial membrane potential.* Rhodamine 123 was used to assess the mitochondrial membrane potential. After treatment with magnolol for 24 h, the cells were washed with 1X PBS and incubated with 1 $\mu$l/ml Rhodamine 123 for 60 min at room temperature in

the dark. The cells were washed with 1X PBS, stained with 1 $\mu$g/ml PI solution and analyzed by flow cytometer (Becton-Dickinson). In this experiment, verapamil (20 $\mu$M) was used as a positive control.

*Western blot analysis.* Cells were treated with magnolol for 24 h. Harvested cells were lysed in protein extraction solution (Intron Biotechnology, Inc., Kyunggi, Korea) containing protease inhibitors and phosphatase inhibitors for 10 min at 4˚C. The total protein concentration in the supernatants was measured by the Bradford assay. After heating at 95˚C for 5 min, total protein samples (40 $\mu$g) were subjected to 6-15% SDS-PAGE. The proteins were transferred onto PVDF membranes (Millipore, Bedford, MA, USA) at 100 V for 60-100 min. The membranes were incubated with 5% BSA in TBST (TBS with 0.05% Tween-20) for 30 min at room temperature and then with primary antibodies diluted (1:200-1:1,000) in 5% BSA in TBST overnight at 4˚C. The membranes were washed three times with TBST and incubated with the corresponding secondary antibodies. Protein bands were detected using an enhanced chemiluminescence detection kit (Intron Biotechnology, Inc.) and a LAS-1000 Imager (Fuji Film Corp., Tokyo, Japan).

*3-Dimensional type I collagen sprouting angiogenesis model.* The three-dimensional tube formation and sprouting angiogenesis model were performed in type I collagen (16,20,31). Briefly, EBs were formed by incubating hanging drop cultures for 3 days and then the EBs were cultured in suspension in EGM-2 medium for 7 days. The EBs were embedded in type I collagen and incubated in EGM-2 medium at 37˚C. Vascular sprouting was induced by incubation with magnolol for 4 days. The morphology of the vascular sprouts was analyzed using a phase contrast microscope (Nikon, Eclipse TE 2000-U, Tokyo, Japan).

*Statistical analyses.* Data are presented as the mean ± SD for the indicated number of independently performed experiments. Statistical significance (P<0.05) was determined using Student's t-test for paired data. Statistical calculations were performed using SPSS for Windows Version 10.0 (SPSS, Chicago, IL, USA).

## Results

*Effects of magnolol on the growth of mES/EB-derived endothelial-like cells.* To determine whether magnolol affects the growth of mES/EB-derived endothelial-like cells, mES/EB-derived differentiated cells (differentiation day 10) were treated with magnolol (0-100 $\mu$M) for 24 h and then cell growth and cytotoxicity were analyzed using the MTT and LDH leakage assays, respectively. As shown in Fig. 1B, the growth inhibition effect of magnolol on the differentiated cells was concentration-dependent. The relatively low concentrations of magnolol (≤25 $\mu$M) did not inhibit the growth of the endothelial cells, but concentrations of magnolol >50 $\mu$M significantly inhibited the growth of these cells (P<0.01). Further experiments confirmed that the growth inhibition activity of concentrations >50 $\mu$M magnolol was associated with the level of cytotoxicity determined by the LDH leakage assay (Fig. 1C).

*Inhibition of vessel formation in the mES/EB-derived endothelial-like cells by magnolol.* Platelet endothelial cell adhesion molecule (PECAM) is considered a cell surface marker for endothelial cells during angiogenesis because PECAM is expressed by endothelial cells and hematopoietic cells (32). To determine whether magnolol inhibits microvessel formation in the mES/EB-derived endothelial-like cells, the cells were treated with magnolol (0-20 $\mu$M) for 24 h and then the expression of the endothelial biomarker PECAM was analyzed. As shown in Fig. 2A, the differentiated (day 10) mES/EB-derived cells exhibited remarkable expression of PECAM, but treatment with magnolol suppressed the mRNA expression of PECAM in a concentration-dependent manner. Magnolol also markedly suppressed the expression of the PECAM protein (Fig. 2B), indicating that magnolol significantly suppressed the expression of PECAM at both the mRNA and protein levels (P<0.05, P<0.01, respectively). The effect of magnolol on the expression of PECAM was also assessed using immunofluorescence in a two-dimensional (2-D) culture of mES/EB-derived differentiated cells. As shown in Fig. 2C, the microvessels generated from the differentiation of mES/EB-derived cells clearly expressed PECAM, but treatment with magnolol (20 $\mu$M) significantly suppressed the expression of PECAM in the differentiated endothelial cells. These results suggest that the anti-angiogenic activity of magnolol is associated with the suppression of PECAM expression. The anti-angiogenic activity of magnolol was also assessed based on the inhibition of vascular tube formation in a three-dimensional (3-D) collagen gel system. In this experiment, vascular tube formation was induced by incubating mES-derived EBs in a type I collagen gel containing EGM-2 medium as previously described by Kim *et al* (20). As shown in Fig. 2D-a, the morphology of the vascular sprouts formed by EB-derived endothelial-like cells was observed after 4 days of induction. Accelerated vascular tube sprouting and an extensive network of cellular outgrowths were observed in the vehicle-treated control cells (Fig. 2D-a) and magnolol effectively blocked tube sprouting and the formation of cellular networks (Fig. 2D-b and -c). These findings suggest that magnolol inhibits vessel formation in mES/EB-derived endothelial-like cells in both 2-D and 3-D culture systems.

*Induction of apoptosis by magnolol.* We next determined whether the inhibition of both tube sprouting and the formation of cellular networks by magnolol is associated with apoptosis in mES/EB-derived endothelial-like cells. Flow cytometric analysis revealed that magnolol treatment resulted in Annexin V-positive cells in a concentration-dependent manner (Fig. 3A). In particular, the percentage of apoptotic cells among the cells treated with 20 $\mu$M magnolol (18.7%) was significantly higher than the percentage of the vehicle-treated control cells (2.6%) (P<0.01). To confirm the association between magnolol treatment and apoptosis, the effects of magnolol on the modulation of apoptotic regulatory molecules were evaluated. As shown in Fig. 3B, the pro-apoptotic protein Bax was upregulated and the anti-apoptotic protein Bcl-xL was downregulated by magnolol treatment. In addition, the activation of procaspase-3 was also induced by magnolol, as confirmed by the increase in the intensity of the cleaved caspase-3 band along with the decrease in the inten-
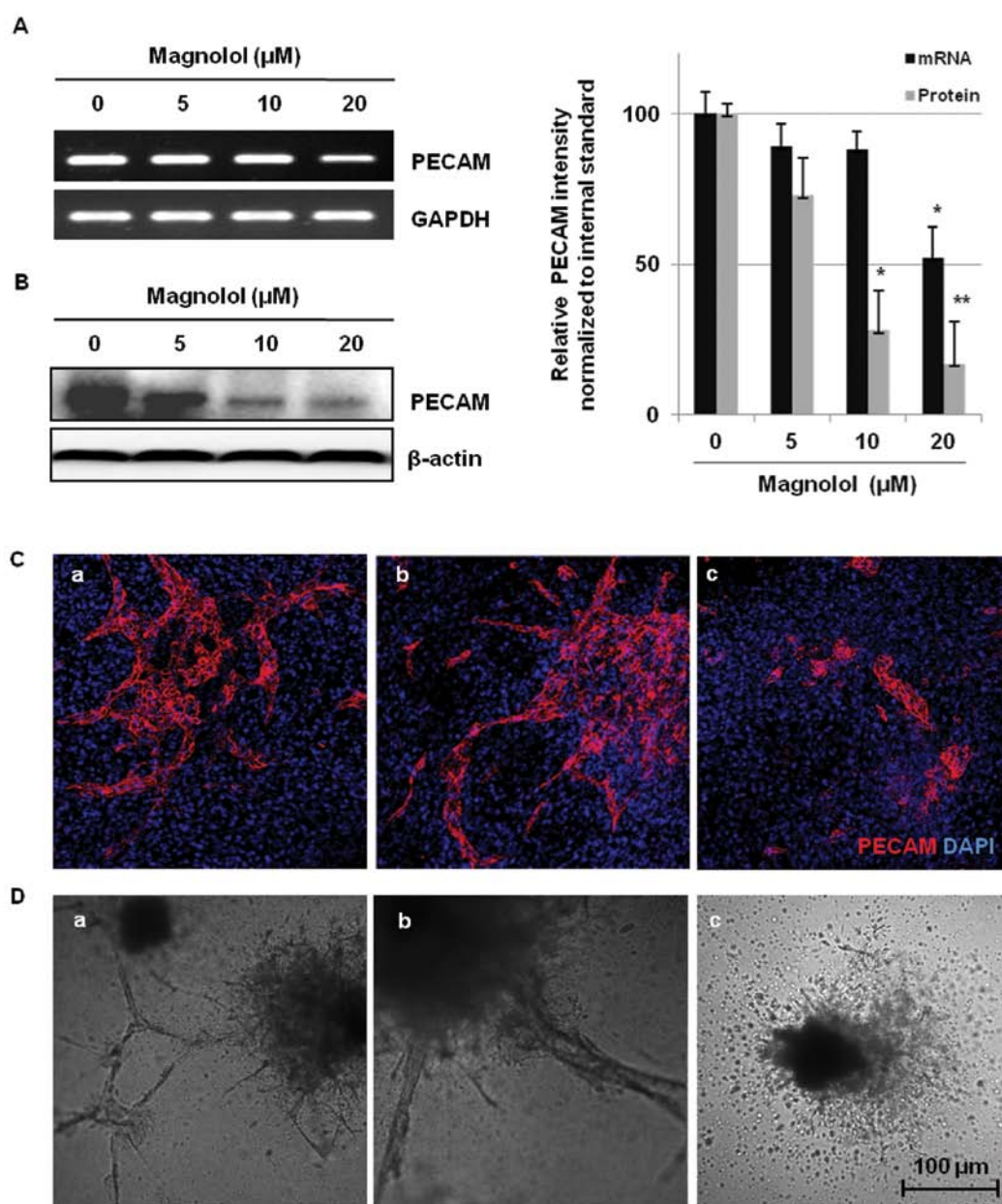
Figure 2. Effect of magnolol on the vascular sprouting of endothelial-like cells derived from mES/EBs. The EBs were differentiated into endothelial cells in EGM-2 containing supplements for 10 days and then treated with magnolol (0-20 $\mu$M) for 24 h. The levels of PECAM mRNA (A) and protein (B) in mES/EB-derived endothelial-like cells were determined by RT-PCR and western blot analysis, respectively. (C) mES/EB-derived endothelial-like cells were immunofluorescently stained with an antibody directed against the endothelial cell biomarker PECAM. Control (a), 5 $\mu$M magnolol (b) and 20 $\mu$M magnolol (c). Magnification, x100. (D) After being cultured in hanging drops for 3 days, EBs derived from mES cells were cultured in suspension for 7 days. The EBs embedded in collagen gels were treated with magnolol for 4 days. The morphology of the endothelial sprouts of EB-derived endothelial-like cells in the control (a) and magnolol-treated samples (b, 5 $\mu$M; and c, 20 $\mu$M) after 4 days of secondary culture in collagen gels. Values indicate the mean ± SD in triplicate tests. The values marked with an asterisk are significantly different from control values, $^*$P<0.05; $^{**}$P<0.01 compared with control. Magnification, x40.

sity of the procaspase-3 band in the western blotting. These findings suggest that magnolol may induce apoptosis of mES/EB-derived endothelial-like cells by upregulating the proapoptotic protein Bax and subsequently activating caspase-3.

*Effects of magnolol on ROS production and the mitochondrial membrane potential.* To further investigate whether the generation of intracellular ROS is involved in the induction of apoptosis by magnolol, the effects of magnolol on the ROS levels were determined using an oxidant-sensitive fluorescent dye in mES/EB-derived endothelial-like cells. Consistent with previous reports (33,34), endogenous production of ROS was detected in mES/EB-derived endothelial-like cells. As shown in Fig. 4A, however, magnolol (20 $\mu$M) significantly increased the intracellular ROS levels (cells exhibited much higher fluorescence intensities) relative to those of the vehicle-treated control cells (P<0.01). We next examined whether the induction of ROS production by magnolol was related to the modulation of the mitochondrial membrane potential in the cells. As shown in Fig. 4B, the active accumulation of Rhodamine 123 was detected in the control cells and the positive control (verapamil-treated) cells. However, treatment
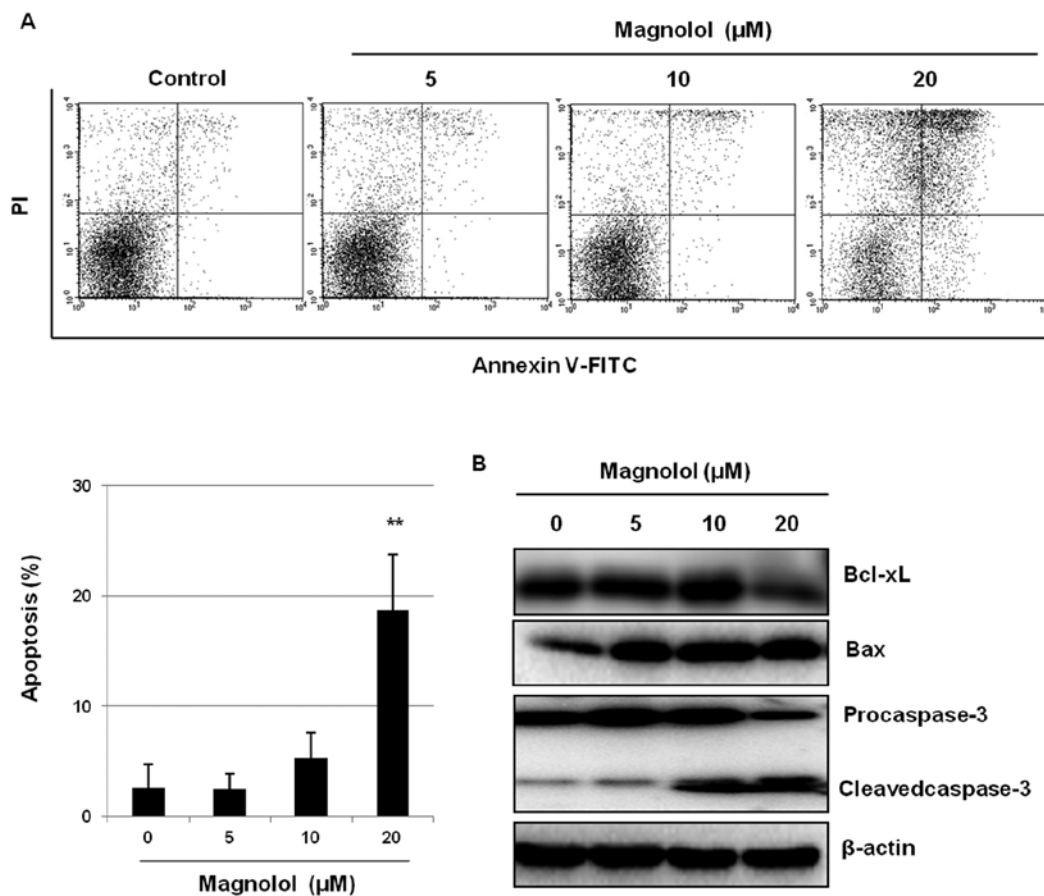
Figure 3. Effect of magnolol on the induction of apoptosis in mES/EB-derived endothelial-like cells. (A) The endothelial differentiated cells were treated with magnolol (0, 5, 10 and 20 µM) for 24 h, stained with Annexin V and propidium iodide (PI) and analyzed on a FACSCalibur flow cytometer. (B) The expression of pro-apoptotic/anti-apoptotic proteins in mES-derived endothelial-like cells was analyzed by western blotting. β-actin was used as an internal control. Data are presented as mean ± SD in triplicate tests. **P<0.01 compared with control.

with magnolol (20 µM) remarkably decreased the uptake of Rhodamine 123 dye in the cells, suggesting that magnolol affects the mitochondrial membrane potential as the result of mitochondrial dysfunction caused by oxidative stress. In addition, the involvement of oxidative stress induced by magnolol in vasculogenesis was also confirmed using the anti-oxidant N-acetyl-L-cysteine (NAC). As shown in Fig. 4C, the suppression of PECAM expression by magnolol (20 µM) was recovered by co-treatment with NAC (5 mM) and magnolol in mES/EB-derived endothelial-like cells. These findings suggest that oxidative stress induced by magnolol in part triggers apoptosis as the result of mitochondrial dysfunction in mES/EB-derived endothelial-like cells.

*Effects of magnolol on MAP kinase signaling.* To further clarify whether the anti-angiogenic activity of magnolol is also mediated by signaling molecules related to cell proliferation, the effects of magnolol on MAPK signaling were determined in mES/EB-derived endothelial-like cells. Magnolol down-regulated the activation of MAP kinases including ERK, JNK and p38 in mES/EB-derived endothelial-like cells (Fig. 5A). The association with MAPK signaling was also confirmed using the inhibitors PD98059 (ERK inhibitor), SP600125 (JNK inhibitor) and SB203580 (p38 inhibitor). As shown in Fig. 5B-D, magnolol suppressed the activation of MAPKs to an extent similar to that of each kinase inhibitor in mES/

EB-derived endothelial-like cells. In addition, the co-treatment of cells with an ERK or JNK inhibitor and magnolol (20 µM) enhanced the suppression of ERK and JNK activation.

*Effects of magnolol on the PI3K/AKT/mTOR signaling pathway.* Accumulating evidence suggests that the PI3K/AKT/mTOR signaling pathway plays an important role in the vasculogenesis and angiogenesis of endothelial cells (35,36). To investigate whether magnolol affects this signaling pathway, the constitutive activation of PI3K/AKT/mTOR expression was evaluated in mES/EB-derived endothelial-like cells. As shown in Fig. 6A, the constitutive activation of both PI3K and PDK was downregulated by treatment with magnolol in a concentration-dependent manner. In addition, the downregulation of AKT activation by magnolol led to the subsequent suppression of signaling by mTOR and its downstream effectors, including S6K and 4EBP1, in mES/EB-derived endothelial-like cells. We also found that co-treatment with the AKT inhibitor LY294002 and magnolol synergistically suppressed the activation of AKT (Fig. 6B).

**Discussion**

Because angiogenesis is associated with many pathological diseases, including cancer, diabetic retinopathy and arthritis, anti-angiogenic agents with unique mechanisms of action
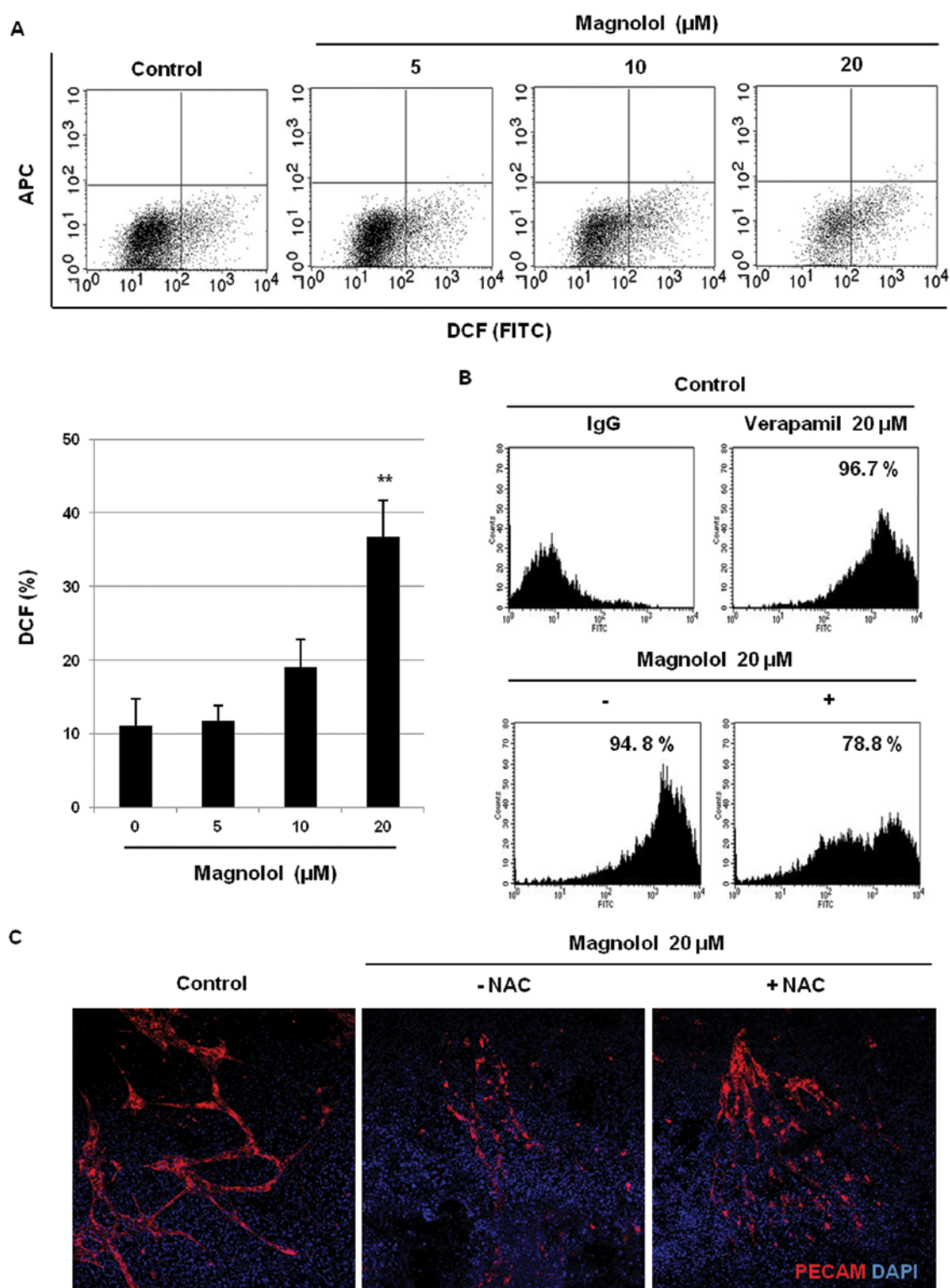
Figure 4. Effect of magnolol on the mitochondrial membrane potential and ROS production in mES/EB-derived endothelial-like cells. (A) Reactive oxygen species (ROS) generation was quantified using $H_2DCFDA$ in mES/EB-derived endothelial-like cells exposed to magnolol (0-20 $\mu$M) for 24 h. (B) After treatment with magnolol or verapamil (positive control) for 24 h, the cells were incubated with Rhodamine 123 and then analyzed by flow cytometer. (C) Cells were treated with magnolol in the absence or presence of 5 mM NAC for 24 h and the cells were stained with PECAM and then analyzed by confocal microscopy. Values indicate the mean ± SD in triplicate tests. The values marked with an asterisk are significantly different from control values; **P<0.01. Magnification, x60.

might be potential candidates to treat or prevent these diseases (37,38). In the present study, we demonstrated that magnolol exerts an anti-angiogenic activity involving ROS-mediated apoptosis and the suppression of the AKT/mTOR signaling pathway in mES/EB-derived endothelial-like cells. A variety of assay systems have been developed to evaluate the anti-angiogenic activity of test compounds (15,20). In particular, the utility of stem cells has been highlighted in recent reports
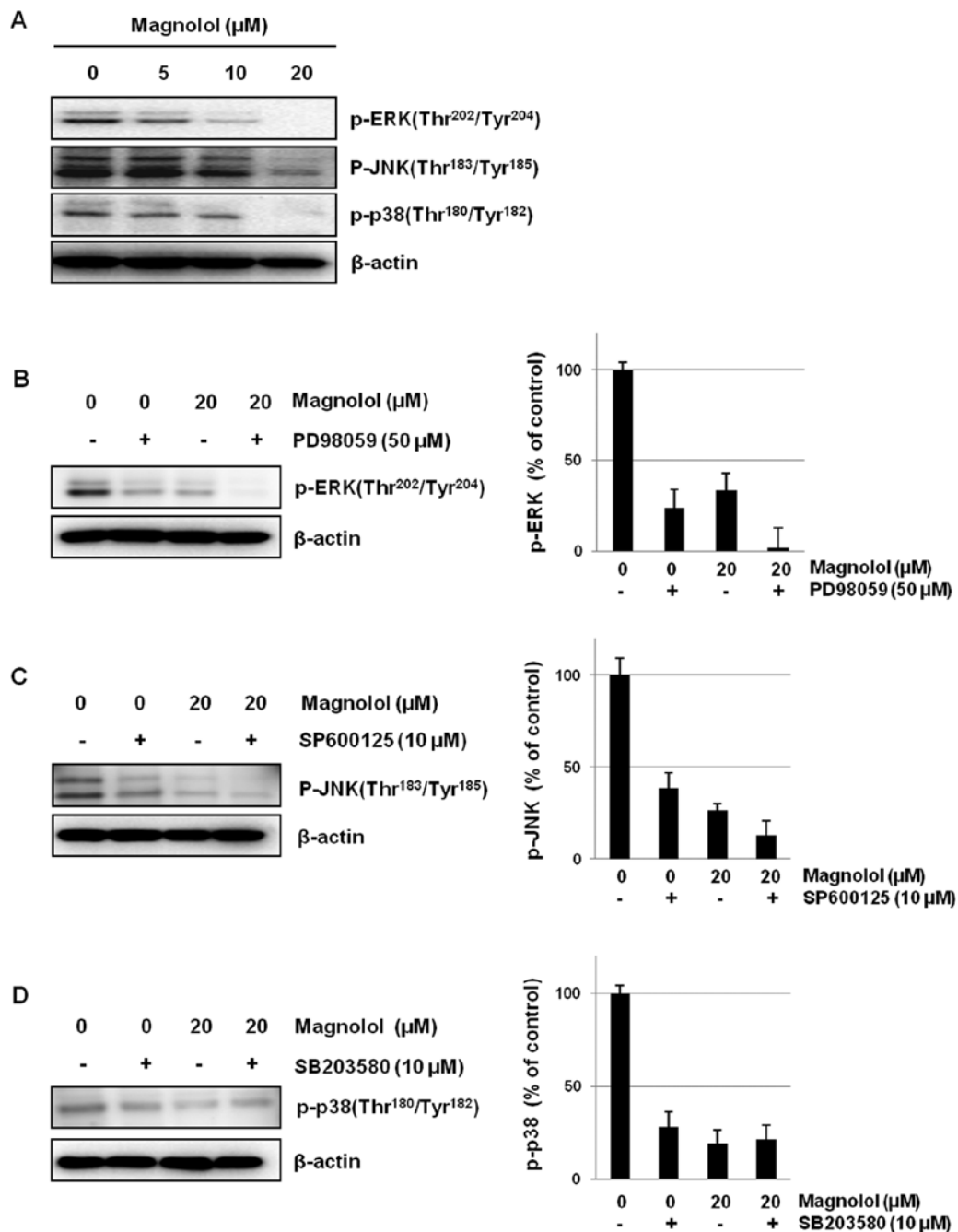
Figure 5. Effect of magnolol on the MAPK signaling pathways in mES/EB-derived endothelial-like cells. (A) Endothelial differentiated cells were treated with magnolol (0, 5, 10, or 20 $\mu$M) for 24 h. Protein samples (40 $\mu$g) were subjected to 10% SDS-PAGE and phosphorylated ERK, JNK and p38 were detected by western blotting. (B-D) The cells were treated with magnolol in combination with MAPK inhibitors and then the activation of ERK, JNK and p38 was detected by western blotting. $\beta$-actin was used as an internal control. Values indicate the mean ± SD in triplicate tests.

(14,15). Recently, we also employed mouse embryonic stem cells and demonstrated that mES/EB-derived endothelial-like cells are a useful *in vitro* model system for the evaluation of the anti-angiogenic activity of natural products (20). Established 2-D and 3-D culture systems using mES/EB-derived endothelial-like cells were used to assess the characteristics of endothelial cells based on the expression of PECAM and the formation of vascular networks. Magnolol effectively inhibited vascular tube formation and suppressed PECAM expression and vessel sprouting in the established endothelial cell systems.

The induction of apoptosis is considered a plausible anti-angiogenic mechanism (39-41). In the present study with mES-derived EBs, we found that magnolol might be able to induce apoptosis in mES/EB-derived endothelial-like cells. The induction of apoptosis by magnolol was confirmed by the increased percentage of Annexin V-positive cells and the enhanced expression of the pro-apoptotic protein Bax. These events subsequently activated caspase-3, thus leading to the induction of apoptosis in the endothelial-like cells (Fig. 3). These findings suggest that the induction of apoptosis by magnolol is in part associated with its anti-angiogenic activity.
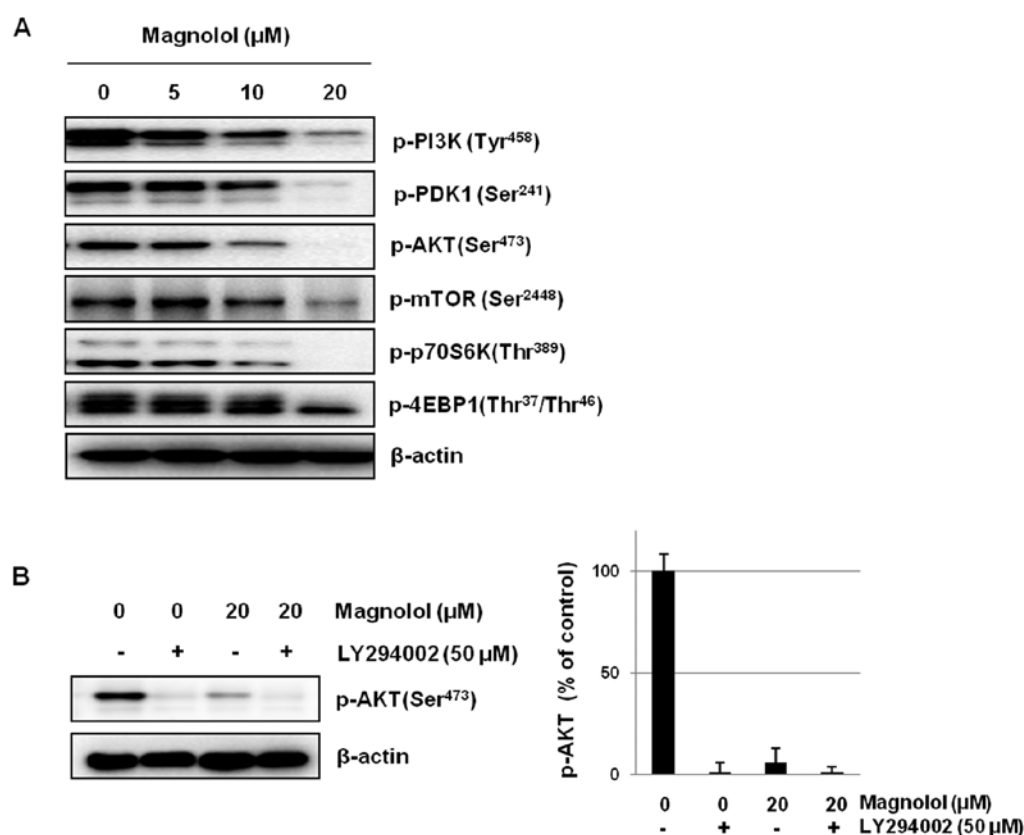
Figure 6. Effects of magnolol on the PI3K/AKT/mTOR signaling pathway in mES/EB-derived endothelial-like cells. (A) Endothelial differentiated cells were treated with magnolol (0, 5, 10, or 20 $\mu$M) for 24 h. Protein samples (40 $\mu$g) were subjected to 6-15% SDS-PAGE and phosphorylated PI3K, p-PDK1, p-AKT, p-mTOR, p-p70S6K and p-4EBP1 were detected by western blotting. (B) The cells were treated with magnolol in combination with the PI3K inhibitor LY294002 and then the level of p-AKT was detected by western blotting. $\beta$-actin was used as an internal control. Values indicate the mean $\pm$ SD in triplicate tests.

Accumulating evidence suggests that reactive oxygen species (ROS) are important mediators in apoptosis. Increased intracellular levels of ROS lead to the activation of apoptosis (42). Based on the finding that magnolol induces apoptosis in mES/EB-derived endothelial-like cells, the association between ROS and the anti-angiogenic activity of magnolol was determined. The cells were analyzed using an oxidant-sensitive fluorescence dye (H$_2$DCFDA) and magnolol treatment (20 $\mu$M) significantly enhanced the production of intracellular ROS in mES/EB-derived differentiated endothelial-like cells. These results indicate that the apoptosis induced by magnolol is due in part to the increased intracellular levels of ROS in the cells. In addition, mitochondria play a crucial role in metabolism and the cell cycle and they coordinate extrinsic and intrinsic signals that affect proliferation, differentiation and cell death. Mitochondria are the major organelles that produce intracellular ROS and mitochondrial dysfunction plays a key role in apoptosis (43). It is also known that increased ROS levels are able to reduce the mitochondrial membrane potential, thus leading to the activation of apoptotic pathways. The Rhodamine 123 dye was employed to assess the membrane potential and we found that magnolol effectively decreased the mitochondrial membrane potential, suggesting that the induction of apoptosis by magnolol is correlated with the increase in intracellular ROS production and the decrease in the mitochondrial membrane potential in mES/EB-derived endothelial-like cells. The association between ROS and the anti-angiogenic activity of magnolol was also partly confirmed by the analysis of PECAM expression when the cells were co-treated with the antioxidant NAC. The treatment with NAC alleviated the ROS-mediated suppression of PECAM expression induced by magnolol, indicating that the anti-angiogenic potential of magnolol might be in part due to the regulation of ROS production in mES/EB-derived endothelial-like cells.

Previous studies have demonstrated that the MAPK signaling pathway is highly involved in cell growth (44) and/or the regulation of the cell cycle (45). In our experiments, magnolol suppressed the activation of MAPKs such as ERK, JNK and p38 and these findings were confirmed using MAPK inhibitors in mES/EB-derived endothelial-like cells. It is well-known that AKT and its downstream signaling partners, including mTOR, are activated during angiogenesis (46). AKT is a serine/threonine kinase that plays a central role in a range of cellular functions, including cell growth, proliferation, migration, protein synthesis, transcription, survival and angiogenesis (9,47). The mTOR kinase, a central regulator of cell metabolism, growth, proliferation and survival, is also activated during various cellular processes, such as tumor initiation, progression and angiogenesis (35). Indeed, the activation of the AKT/mTOR signaling pathway in endothelial cells promotes the survival of these cells when cultured in vitro (36). In the present study, we found that magnolol suppressed the phosphorylation of AKT, mTOR, S6K and 4EBP1 in mES/EB-derived endothelial-like cells. These suppressive effects of magnolol might be an additional mechanism of action explaining this compound's anti-angiogenic activity in mES/EB-derived endothelial-like cells.

In conclusion, the present findings demonstrate for the first time that magnolol inhibits angiogenesis in mES/EB-derived endothelial-like cells by regulating ROS-mediated apoptosis and inhibiting the PI3K/AKT/mTOR signaling pathway.

## Acknowledgements

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