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Antrodia cinnamomea produces anti-angiogenic effects by inhibiting the VEGFR2 signaling pathway

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ABSTRACT

Ethnopharmacological relevance: The medicinal mushroom *Antrodia cinnamomea* has been used to treat cancer but its anti-angiogenic effects have not been studied in detail.
Aim of the study: The main objective of this study was to determine the molecular mechanism of activity underlying the anti-angiogenic effects of *A. cinnamomea.*
Materials and methods: The effects of an *A. cinnamomea* ethanol extract (ACEE) on cell migration and microvessel formation were investigated in endothelial cells *in vitro* and Matrigel plugs implanted into mice *in vivo*. Activation of intracellular signaling pathways was examined using Western blotting. Protein expression was assessed using immunohistochemistry in a mouse model of lung metastasis.
Results: We show that treatment with ACEE inhibits cell migration and tube formation in human umbilical vein endothelial cells (HUVECs). ACEE suppresses phosphorylation of vascular endothelial growth factor receptor 2 (VEGFR2) and expression of pro-angiogenic kinases in vascular endothelial growth factor (VEGF)-treated HUVECs, in addition to reducing expression of Janus kinase 2 (JAK2) and phosphorylation of signal transducer and activator of transcription 3 (STAT3). ACEE treatment inhibits VEGF-induced microvessel formation in Matrigel plugs *in vivo*. In addition, ACEE significantly reduces VEGFR2 expression in Lewis lung carcinoma cells and downregulates the expression of cluster of differentiation 31 (CD31) and VEGFR2 in murine lung metastases.
Conclusion: These results indicate that *A. cinnamomea* produces anti-angiogenic effects by inhibiting the VEGFR2 signaling pathway.

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1. Introduction

Angiogenesis refers to the formation of new blood vessels from a preexisting vascular network, a process that is essential for the development and progression of malignant tumors and metastases (Kerbel and Folkman, 2002). Although numerous factors have been implicated in tumor angiogenesis, vascular endothelial growth factor (VEGF) is among the most critical mediators that promote angiogenesis (Ferrara and Kerbel, 2005). VEGF exerts its functions by binding to VEGF receptors (VEGFRs) and activating downstream signaling pathways. VEGFR2 is the main effector of VEGF-induced angiogenic processes that include cell proliferation, vascular permeability, cell migration and cell survival (Holmes et al., 2007). Activation of VEGFR2 activates downstream signaling molecules, including the Src family kinase, focal adhesion kinase (FAK), phospholipase C gamma (PLCγ), serine/threonine protein kinase (i.e., Akt) and extracellular signal-regulated kinase (ERK) (Olsson et al., 2006). In addition, VEGFR2 signaling activates Janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3), which in turn affects cell survival, migration and tube formation (Dong et al., 2010). Inhibition of STAT3 signaling reduces angiogenesis, tumor growth and metastasis (Kim et al., 2013; Yuan et al., 2015). Therefore, the VEGFR2 signaling pathway is a potential therapeutic target for treating cancer and angiogenesis-related diseases.

Traditional Chinese medicine has a long history of use in Asian countries to improve health and longevity. Medicinal mushrooms such as *Agaricus blazei* Murrill, *Ganoderma lucidum* and *Ophiocordyceps sinensis* produce many beneficial effects on animals and humans, including immunomodulatory, antidiabetic, anti-fatigue and anti-cancer effects (Wasser, 2014; Martel et al., 2017a, 2017b, 2017c). We showed earlier that a water extract of *G. lucidum* mycelium produces anti-inflammatory, anti-diabetic and anti-obesogenic effects in high-fat diet-fed mice (Chang et al., 2015; Holmes, 2015). These effects were transferable to high-fat diet-fed mice by fecal transplantation from *G. lucidum*-treated mice, indicating that the effects were mediated by the gut microbiota. We also observed that an ethanol extract of *Hirsutella sinensis* mycelium (the anamorph of *O. sinensis*) reduces pulmonary inflammation and fibrosis in bleomycin-treated mice (Huang et al., 2015). These medicinal mushrooms thus represent a valuable source of bioactive compounds for disease prevention and drug development.

Antrodia cinnamomea is a medicinal mushroom that grows on the aromatic tree *Cinnamomum kanehirai* in Taiwan. Also known as *Antrodia camphorata* or *Taiwanofungus camphorata*, the fungus has a long history of use by aboriginal people for the treatment of diarrhea, abdominal pain, hypertension, itchy skin and cancer (Geethangili and Tzeng, 2011). Whole extracts and active compounds of *A. cinnamomea* produce various biological activities, such as reduction of inflammation (Huang et al., 2014), liver protection (Ao et al., 2009), immunomodulation (Chen et al., 2008) and antioxidant effects (Song and Yen, 2002). Previous studies have shown that *A. cinnamomea* promotes anticancerogenic activity in cancer of the liver, prostate, bladder, lung and breast (Chiang et al., 2010; Chen et al., 2007; Peng et al., 2007; Yang et al., 2006; Huang et al., submitted for publication). In addition, polysaccharides isolated from *A. cinnamomea* inhibit angiogenesis in bovine endothelial cells (Cheng et al., 2005) and human endothelial cells (Yang et al., 2009; Cheng et al., 2011). However, the molecular mechanism underlying the anti-angiogenic activity of the fungus remains unclear.

In the present study, we prepared an *A. cinnamomea* ethanol extract (ACEE) and examined the molecular mechanism underlying its anti-angiogenic activity *in vitro* and *in vivo*. Of note, we observed that *A. cinnamomea* inhibits angiogenesis by reducing activation of the VEGFR2-STAT3 signaling pathway.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), Medium 199 (M199), Opti-MEM, hydroxyethyl piperazineethanesulfonic acid (HEPES) buffer, sodium pyruvate, recombinant human VEGF, penicillin and streptomycin were purchased from Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT, USA). Primary antibodies against Akt, phospho-Akt (P-Akt, Ser473), FAK, phospho-FAK (P-FAK, Tyr397), PLCγ1, phospho-PLCγ1 (P-PLCγ1, Ser1248), Src, phospho-Src (P-Src, Tyr416), STAT3, phospho-STAT3 (P-STAT3, Tyr705), VEGFR2, phospho-VEGFR2 (P-VEGFR2, Tyr1175), and JAK2 were purchased from Cell Signaling Technology (Beverly, MA, USA); anti-CD31, anti-VEGFR2, anti-VEGF and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and anti-mouse antibodies were obtained from Santa Cruz Biotechnology.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs; BCRC H-UV001) and murine Lewis lung carcinoma cells (LLC; BCRC-60050) were obtained from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). HUVECs were grown in M199 medium containing endothelial cell growth medium (EGM) − 2 SingleQuots Kit (containing FBS, VEGF and other growth factors) (Lonza, Basel, Switzerland), 1 mM sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin. LLC cells were maintained in DMEM supplemented with 10% heat-inactivated FBS, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin. All cell lines were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Preparation of ACEE

A. cinnamomea was isolated and characterized by Chang Gung Biotechnology Corporation (Taipei, Taiwan). Species identification was based on sequencing of 5.8 S rDNA and internal transcribed spacers (ITS-1 and ITS-2) as described before (Huang et al., 2014). ACEE was prepared as described before (Huang et al., 2014), with minor modifications to obtain a concentrated extract. Briefly, 400 g of dried *A. cinnamomea* mycelium powder was mixed with 10 l of 95% ethanol (v/v). The mixture was incubated with agitation at 80 °C for 1 h, before centrifugation at 5900*g* for 30 min at 25 °C. The supernatant was concentrated using a vacuum concentrator at 65 °C to obtain ~80 g of mixture. The mixture was centrifuged again as above, and the supernatant representing ACEE was collected (~40 g).

2.4. Analysis of ACEE composition

Triterpenoids (34.3%, w/w) were measured in ACEE as described before (Fan and He, 2006). Pinicolol B (91 ppm) was measured as before (Wu et al., 2017) using high-performance liquid chromatography (HPLC) on a Cosmosil 5C18-MS-II column (4.6 × 250 mm, 5 μm) with a mobile phase of 0.009% phosphoric acid (solution A) and acetonitrile (solution B). A gradient of solutions A and B was used as eluent. At time 0, the gradient of solutions A/B in the mobile phase was 70/30. From 0–110 min, the gradient of solutions A/B was 53/47, followed by 0/100 at 110–170 min. The Waters 996 Photodiode array detector was used at a wavelength of 243 nm and the flow rate used was 1 ml/min. Chemical analysis of ACEE was performed using standard chemistry procedures (SuperLab, New Taipei City, Taiwan).

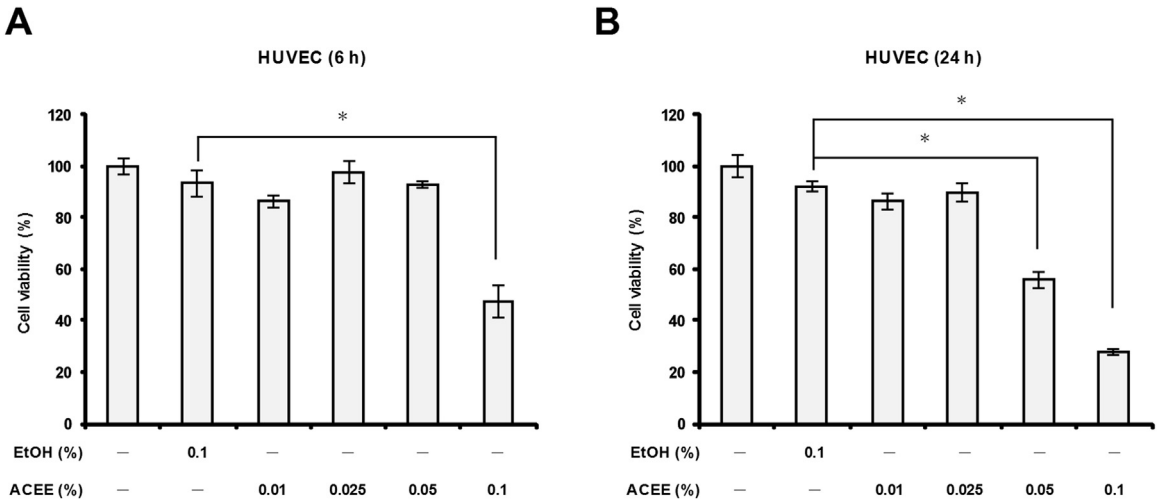

Fig. 1. Effect of ACEE on endothelial cell viability. HUVECs were treated with control ethanol (EtOH, 0.1%) or ACEE (0.01–0.1%) for (A) 6 h or (B) 24 h, and cell viability was analyzed using the MTT assay. Data are presented as means ± SEM of three experiments performed in duplicate. * $P < 0.05$ versus control ethanol-treated cells.

2.5. Cell viability assay

The dimethylthiazol diphenyltetrazolium bromide (MTT) assay was used to evaluate cell viability, as described previously (Huang et al., 2014). HUVECs (2×10^4 cells/well) were seeded in 96-well plates and cultivated under normal cell culture conditions for 24 h. Cells were treated with ACEE (0.01%, 0.025%, 0.05%, and 0.1%) for 6 or 24 h.

2.6. In vivo Lewis lung carcinoma model

Eight-week-old male C57BL/6 mice (22–25 g) were purchased from the National Laboratory Animal Center (Taipei, Taiwan). Mice were housed in an air-conditioned animal facility under constant temperature and humidity with a 12 h/12 h light/dark cycle. Food and water were given *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Chang Gung University (Taoyuan, Taiwan; Document CGU14–132), and the experiments were performed in accordance with the guidelines. After a one-week adaptation period, mice were randomly divided into four groups (n = 5 mice/group): group 1 (control) was treated with 1% ethanol from day 2; group 2 was treated with 0.5% ACEE from day 2; group 3 was treated with 1% ACEE from day 2; group 4 was treated with 1% ACEE from day 15. A suspension of LLC cells (2.5×10^5 cells in 50 µl of Opti-MEM) was inoculated into the right hind paw of each mouse on day 1. The vehicle (1% ethanol dissolved in 0.1 ml of sterile phosphate-buffered saline, PBS) and ACEE (0.5% or 1%, dissolved in 0.1 ml of sterile PBS) were administered by oral gavage five times per week, starting one day after cancer cell injection. On day 45, all mice were sacrificed.

2.7. Immunohistochemistry

Immunohistochemistry was performed based on an established protocol (Huang et al., 2015), with modifications. Briefly, tissue samples from the lungs of sacrificed mice were collected and immediately fixed with 4% paraformaldehyde before embedding with paraffin wax and routine processing. Serial paraffin sections (4 µm) were prepared using a rotator microtome. Immunohistochemical staining of paraffin-embedded sections was performed using the EnVision Detection Systems (Dako, Glostrup, Denmark) according to the manufacturer's instructions. Antigen retrieval was performed by boiling slides in 10 mM sodium citrate buffer (pH 6) for 10 min. Primary antibodies against CD31, VEGF and VEGFR2 were applied overnight at 4 °C. Slides were counterstained with hematoxylin, dehydrated and mounted. Slides were observed by two pathologists in a blind manner. Images from stained slides were acquired using HistoFAXS (Tissue Gnostics, Vienna, Austria).

2.8. Enzyme-linked immunosorbent assay (ELISA)

The concentrations of VEGF in ACEE-treated LLC culture medium were measured using a commercial mouse VEGF ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

2.9. Wound healing assay

HUVECs were grown to full confluence in 6-well plates and starved with M199 basal medium for 6 h. A wound was made by scratching with a sterile 200-µl pipette tips and washed with PBS. M199 supplemented with EGM-2 SingleQuots and ethanol (0.05%) or ACEE at various concentrations (0.01%, 0.025%, and 0.05%) was added into the wells. After 16 h of incubation at 37 °C, viable cells were detected using calcein acetoxylmethyl (AM) (Invitrogen, Carlsbad, CA, USA). Images were taken at 485/538 nm (ex/em) using a fluorescence microscope (Nikon). Cells that had migrated were counted from three randomly selected fields. Cells that had received only ethanol (0.05%) served as a vehicle control. Inhibition percentage was expressed as percentage of vehicle control, which was set at 100%. The assay was repeated independently three times.

2.10. Tube formation assay

Matrigel basement membrane matrix (BD Biosciences, San Jose, CA, USA) was thawed at 4 °C, pipetted (10 µl) into a pre-chilled 15-well Ibidi µ-slide (Ibidi GmbH, Munich, Germany), and incubated at 37 °C for 30 min. HUVECs (1×10^4 cells/well) were seeded on Matrigel and treated with the vehicle (0.05% ethanol) or ACEE (0.01%, 0.025%, and 0.05%). After incubation at 37 °C for 6 h, viable cells were detected using calcein AM (Invitrogen). Network-like structures formed by endothelial cells were photographed using a fluorescence microscope (Nikon) and the extent of tube formation was evaluated by measuring total tube area per field.

2.11. Matrigel plug assay

Matrigel (0.5 ml) containing VEGF (200 ng/ml), heparin (50 units), and the vehicle (1% ethanol) or ACEE (0.5% or 1%) was injected subcutaneously into the ventral area of C57BL/6 mice. Five mice were

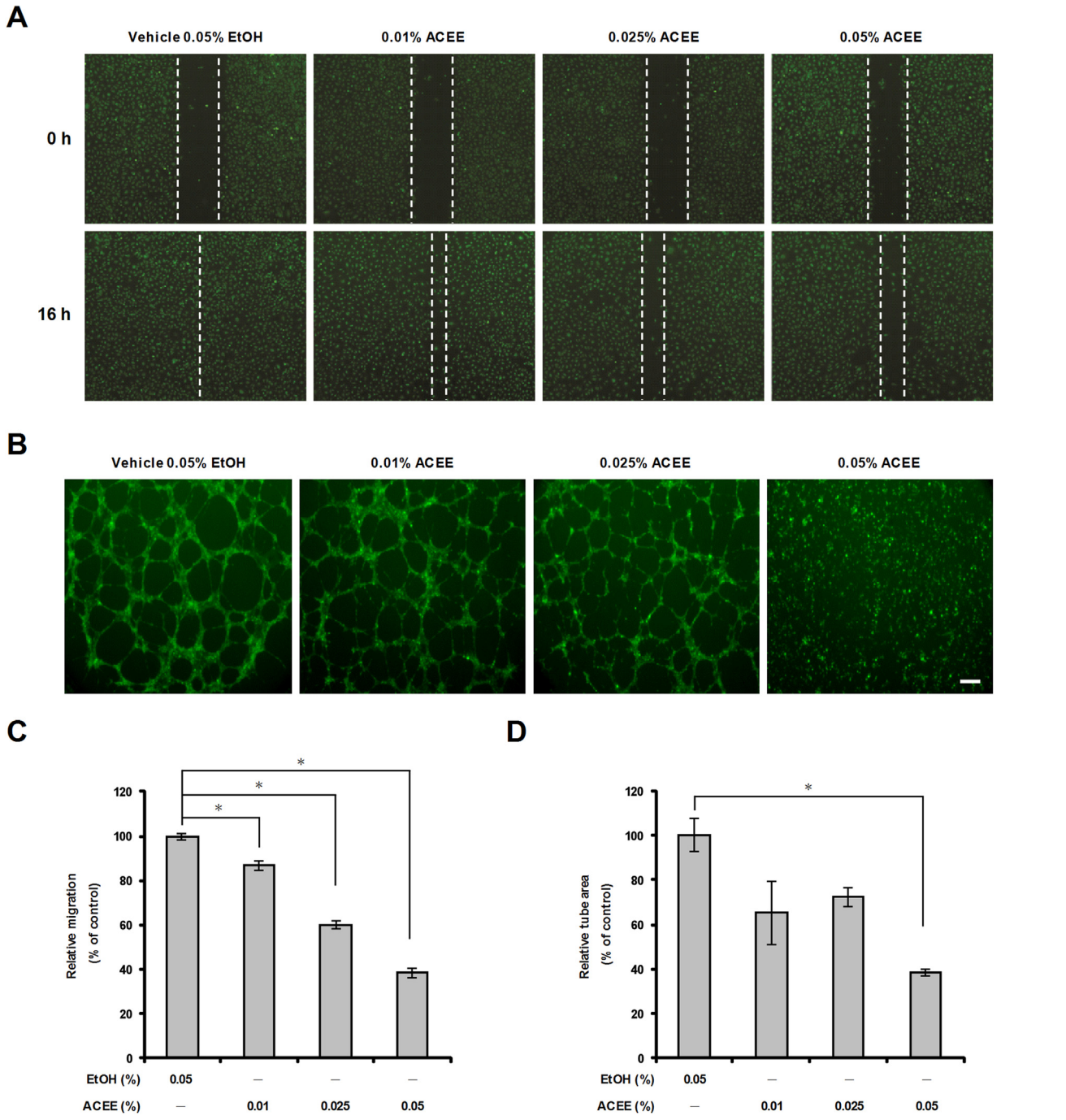

Fig. 2. ACEE inhibits cell migration and tube formation in endothelial cells. (A) ACEE inhibits HUVEC migration in the wound healing assay. Cells grown to confluence were scratched with a pipette tip and treated with vehicle ethanol (EtOH, 0.05%) or ACEE (0.01–0.05%). After 16 h of incubation, representative fields were photographed. (B) ACEE inhibits tube formation in HUVECs. After treatment with vehicle or ACEE for 6 h, tubular structures were photographed. Scale bar = 100 μm. (C) Cell migration levels following ACEE treatment in HUVECs. (D) Effect of ACEE on tube formation in HUVECs. Data are presented as means ± SEM of three independent experiments. * $P < 0.05$ versus control ethanol-treated cells.

used for each group. After 30 days, mice were euthanized, and plugs were removed. The amount of hemoglobin was measured using a Drabkin's reagent kit according to the manufacturer's instructions (Sigma-Aldrich).

2.12. Western blot analysis

Protein extraction and Western blot analysis were performed as described (Huang et al., 2014). Briefly, an equal amount of protein was loaded and electrophoresed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat milk in Tris-buffered saline-Tween 20 (TBS-T; 0.1% Tween 20 in 1 × TBS, pH 7.4) for 1 h at room temperature, followed by incubation overnight with primary antibodies at 4 °C. After washing steps, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Finally, the blots were visualized using enhanced chemiluminescence (Millipore, Billerica, MA, USA) and quantified by densitometry.

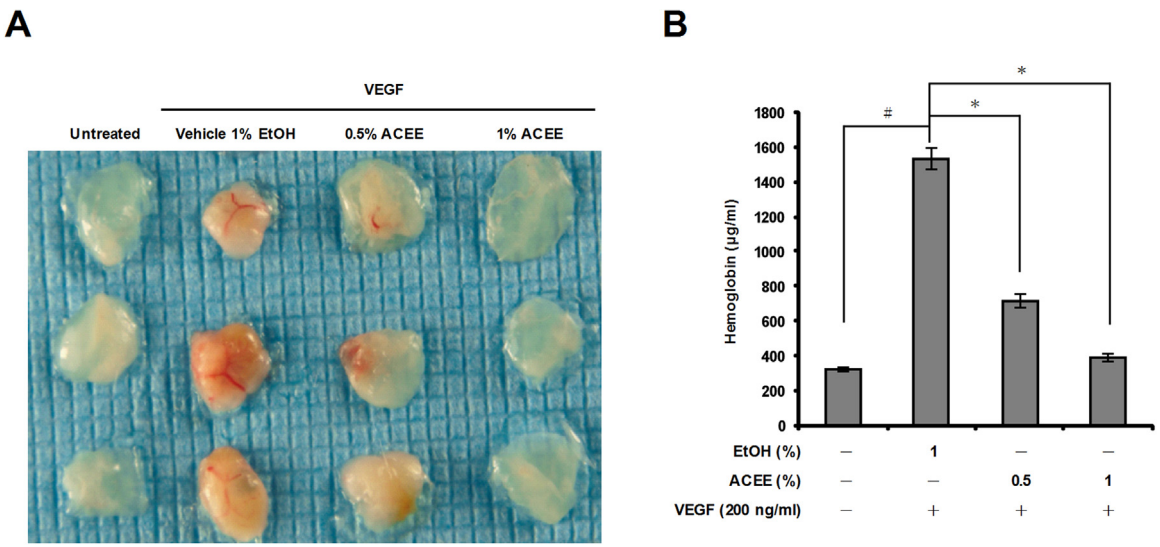

Fig. 3. ACEE inhibits VEGF-induced angiogenesis *in vivo*. Mice were injected with 0.5 ml of Matrigel containing ACEE (0.5% or 1%), VEGF (200 ng/ml), and heparin (50 units) into the ventral area. Matrigel containing ethanol (EtOH, 1%) instead of ACEE was used as a control. After 30 days, the skin was dissected and Matrigel plugs were removed. (A) Representative pictures of Matrigel plugs. (B) Quantification of hemoglobin levels in Matrigel plugs. Data are presented as means ± SEM. (n = 5). $^{\#}P < 0.05$ versus untreated group. $^{*}P < 0.05$ versus control ethanol-treated group.

2.13. Statistical analysis

Results are shown as means ± standard error of the mean (SEM) from at least three independent experiments with duplicates for each condition. Comparisons for multiple groups were done using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Differences between two means were evaluated using the two-tailed Student's *t*-test. A P value < 0.05 was considered statistically significant.

3. Results

3.1. ACEE inhibits cell migration and tube formation in endothelial cells

We examined the effects of ACEE on the viability of HUVECs using the MTT assay. Cell viability was not significantly affected by treatment with 0.01 or 0.025% of ACEE for 6 h or 24 h (Fig. 1A and B). However, incubation with 0.1% ACEE for 6 h reduced cell viability by approximately 50% (Fig. 1A). Cell viability was also reduced following 24 h of treatment with 0.05% and 0.1% of the extract (Fig. 1B). We selected ACEE concentrations and treatment duration producing no effect on cell viability for further experiments.

Given that cell migration is a key step during angiogenesis (Patan, 2004), we used the wound healing assay to monitor the effects of ACEE on endothelial cell motility. ACEE inhibited HUVEC migration in a dose-dependent manner, as assessed 16 h after wounding (Fig. 2A and C). We also examined the formation of capillary tubes by endothelial cells, another critical process during angiogenesis (Patan, 2004). HUVECs were cultivated onto Matrigel in the presence of ACEE at various concentrations (0.01%, 0.025%, and 0.05%), and the formation of capillary-like structures was examined. HUVECs showed well-formed tubular structures in the control vehicle group (Fig. 2B, 0.05% ethanol, EtOH). ACEE affected the formation capillary structures (Fig. 2B) and almost 60% of capillary-like structures was abolished when HUVECs were incubated with ACEE at 0.05% for 6 h (Fig. 2D). These results demonstrate that ACEE inhibits cell migration and tube formation in endothelial cells.

3.2. ACEE inhibits VEGF-induced angiogenesis in vivo

We used the Matrigel plug assay to examine the effects of ACEE on angiogenesis *in vivo*. In this assay, Matrigel containing VEGF (200 ng/ml) plus either ethanol (1%) or ACEE (0.5% or 1%) were injected subcutaneously into the ventral area of mice, and blood vessel formation was assessed 30 days later by measuring hemoglobin levels. As shown in Fig. 3A, Matrigel plugs in the control vehicle group that was supplemented with VEGF alone appeared red, indicating microvessel formation. By contrast, addition of ACEE (0.5% or 1%) in the Matrigel plugs considerably inhibited VEGF-induced vessel formation (Fig. 3A). Quantification of angiogenesis levels based on hemoglobin content showed that ACEE significantly inhibited VEGF-induced angiogenesis compared with the control ethanol-treated group (Fig. 3B). These results show that ACEE inhibits angiogenesis *in vivo*.

3.3. ACEE reduces VEGF-induced VEGFR2 signaling activity in endothelial cells

VEGF-induced VEGFR2 phosphorylation leads to activation of several downstream signaling effectors that are responsible for endothelial cell survival, proliferation, migration and tube formation (Olsson et al., 2006). We therefore examined whether ACEE affects VEGFR2 phosphorylation in VEGF-treated cells. Expression of total VEGFR2 and phosphorylated VEGFR2 (P-VEGFR2) was assessed by Western blot analysis. As shown in Fig. 4A and B, ACEE at concentrations ranging from 0.01% to 0.05% inhibited VEGFR2 phosphorylation in HUVECs in a dose-dependent manner. In contrast, total VEGFR2 protein levels were not affected by ACEE treatment (Fig. 4A). To evaluate the molecular mechanism underlying the inhibitory effects of ACEE on angiogenesis, we examined signaling proteins that act downstream of VEGFR2 activation. Our results showed that ACEE reduced VEGF-induced phosphorylation of angiogenic kinases (P-PLCγ1, P-FAK, P-Src and P-Akt), whereas total protein levels were unaffected (Fig. 4C–H). Taken together, our results reveal that ACEE blocks angiogenesis by suppressing VEGFR2-associated signaling pathways.

3.4. ACEE reduces JAK2 expression and STAT3 phosphorylation in endothelial cells

Previous studies suggest that VEGF triggers activation of STAT3 signaling in HUVECs (Dong et al., 2010). We therefore investigated whether ACEE modulates STAT3 phosphorylation and activation. Western blot analysis revealed that ACEE reduced the level of

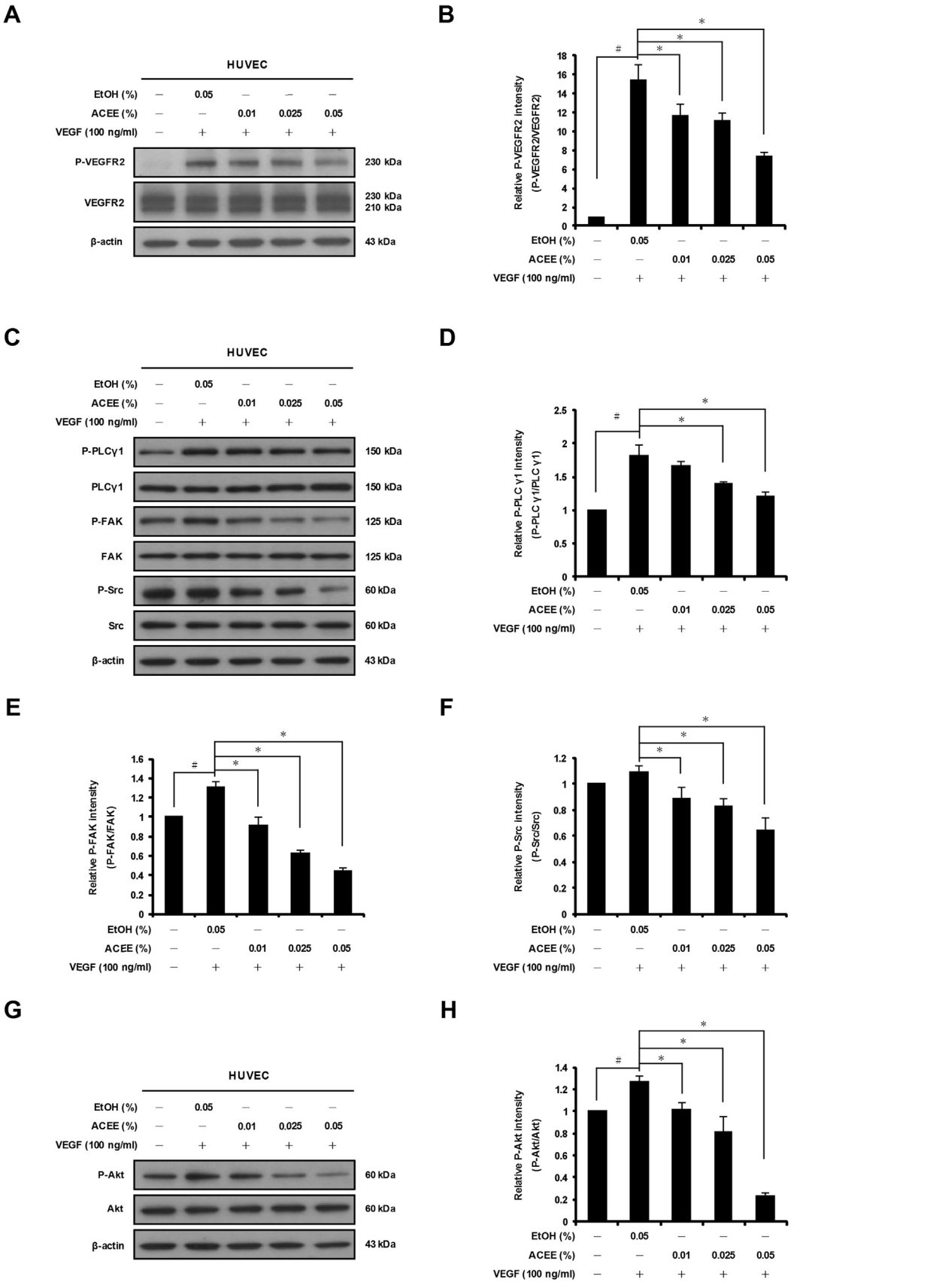

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Fig. 4. ACEE reduces VEGFR2 phosphorylation and downstream signaling in VEGF-treated HUVECs. Cells were starved in serum-free medium for 6 h, pretreated with ACEE (0.01–0.05%) for 1 h, and treated with VEGF (100 ng/ml) for 10 min (VEGFR2, PLCγ1, FAK, and Src) or 30 min (Akt) before protein extraction and Western blot analysis. (A) ACEE inhibits VEGFR2 phosphorylation in VEGF-induced HUVECs. (B) Quantification of P-VEGFR2 levels following normalization against total VEGFR2. (C) ACEE suppresses phosphorylation of PLCγ1, FAK, and Src in VEGF-induced HUVECs. Quantification of P-PLCγ1 (D), P-FAK (E), and P-Src (F) levels normalized against total proteins. (G) ACEE inhibits Akt phosphorylation in VEGF-induced HUVECs. (H) Levels of P-Akt after normalization against total Akt protein levels. Data are presented as means ± SEM of three experiments performed in duplicate. $^{\#}P < 0.05$ versus untreated cells. $^{*}P < 0.05$ versus control ethanol-treated cells.

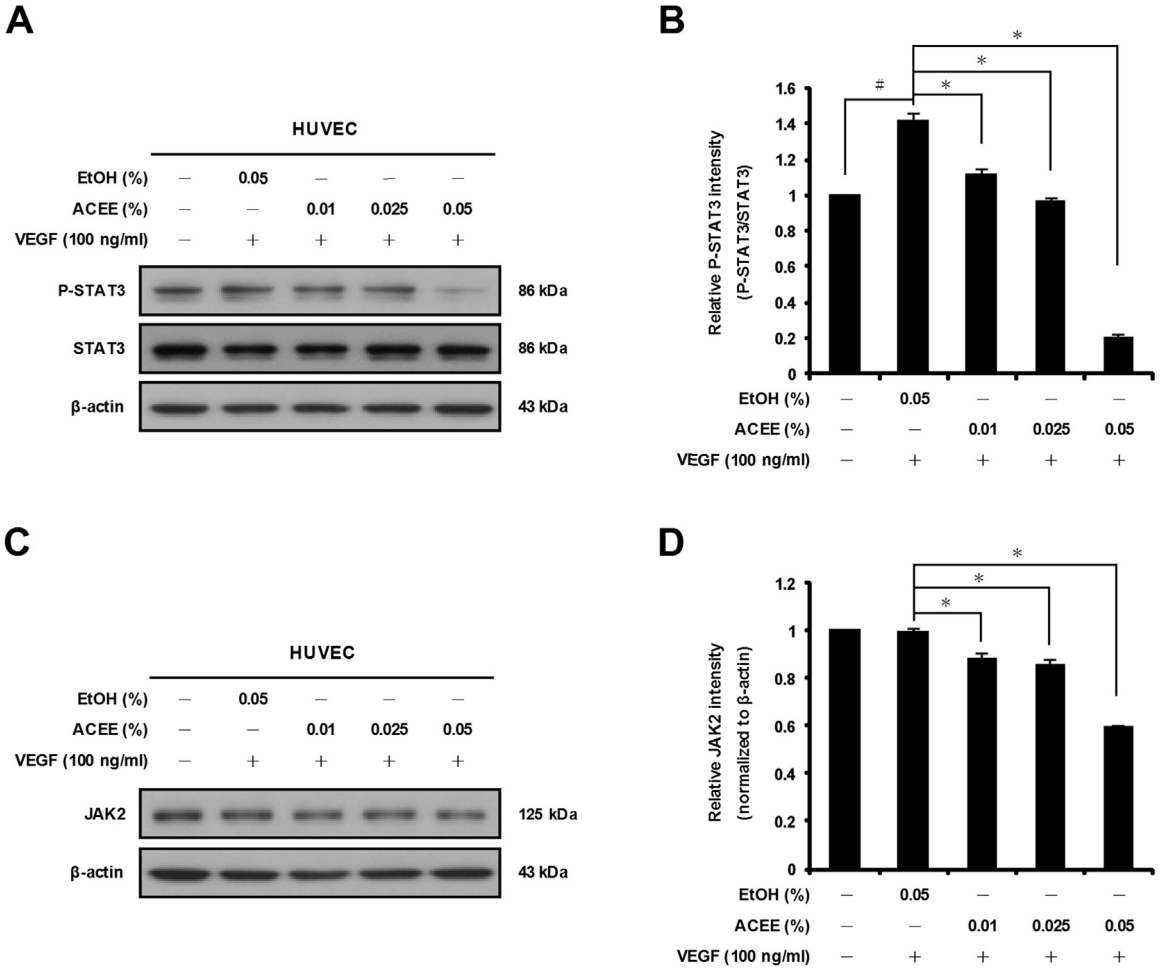

Fig. 5. ACEE reduces JAK2 expression and STAT3 phosphorylation in VEGF-treated HUVECs. Cells were starved in serum-free medium for 6 h, pretreated with ACEE for 1 h, and treated with VEGF (100 ng/ml) for 2 h. Proteins were extracted and Western blotting was performed using anti-JAK2, anti-P-STAT3, anti-STAT3, or β-actin antibodies. (A) ACEE suppresses VEGF-induced phosphorylation of STAT3 in HUVECs. (B) Quantification of P-STAT3 levels after normalization against total STAT3 protein levels. (C) ACEE reduces expression of JAK2 in HUVECs. (D) Densitometry analysis of JAK2 protein expression normalized to β-actin. Data are presented as means ± SEM of three experiments performed in duplicate. $^{\#}P < 0.05$ versus untreated cells. $^{*}P < 0.05$ versus control ethanol-treated cells.

phosphorylated STAT3 (P-STAT3) in a dose-dependent manner in HUVECs (Fig. 5A and B). Given that STAT3 activation is primarily mediated by JAK2 (Dong et al., 2010; Liu et al., 2011), we examined JAK2 expression in ACEE-treated HUVECs. ACEE dose-dependently suppressed JAK2 expression in HUVECs compared with the control VEGF-treated vehicle (Fig. 5C and D). These findings indicate that ACEE effectively suppresses the phosphorylation and activation of STAT3 and the expression of JAK2 in HUVECs.

3.5. ACEE inhibits VEGFR2 expression in lung cancer cells

Constitutive activation of the JAK2/STAT3 signaling pathway has been shown to play an important role in lung tumor angiogenesis (Zhao et al., 2011). We observed previously that *A. cinnamomea* induces apoptosis in LLC cells by repressing the JAK2/STAT3 signaling pathway (Huang et al., submitted for publication). To explore whether the anti-angiogenic effects of ACEE involves modulation of VEGF expression in lung cancer cells, we examined VEGF expression in LLC cells in response to ACEE treatment. LLC cells were treated with ACEE for 6 h, and VEGF protein level was determined using Western blot analysis. As shown in Fig. 6A and B, ACEE treatment from 0.01% to 0.05% did not affect VEGF expression levels, whereas ACEE treatment at 0.1% significantly upregulated VEGF expression. Given that VEGF induces angiogenic activity mainly by activating VEGFR2, we examined VEGFR2 protein expression in LLC cells in response to ACEE. ACEE reduced VEGFR2 expression in a dose-dependent manner in LLC cells (Fig. 6C and D). ACEE treatment led to a dose-dependent increase of VEGF secretion by LLC cells, as assessed by ELISA (Fig. 6E). Taken together, these results suggest that ACEE produces anti-angiogenic effects by inhibiting VEGFR2 expression in LLC cells.

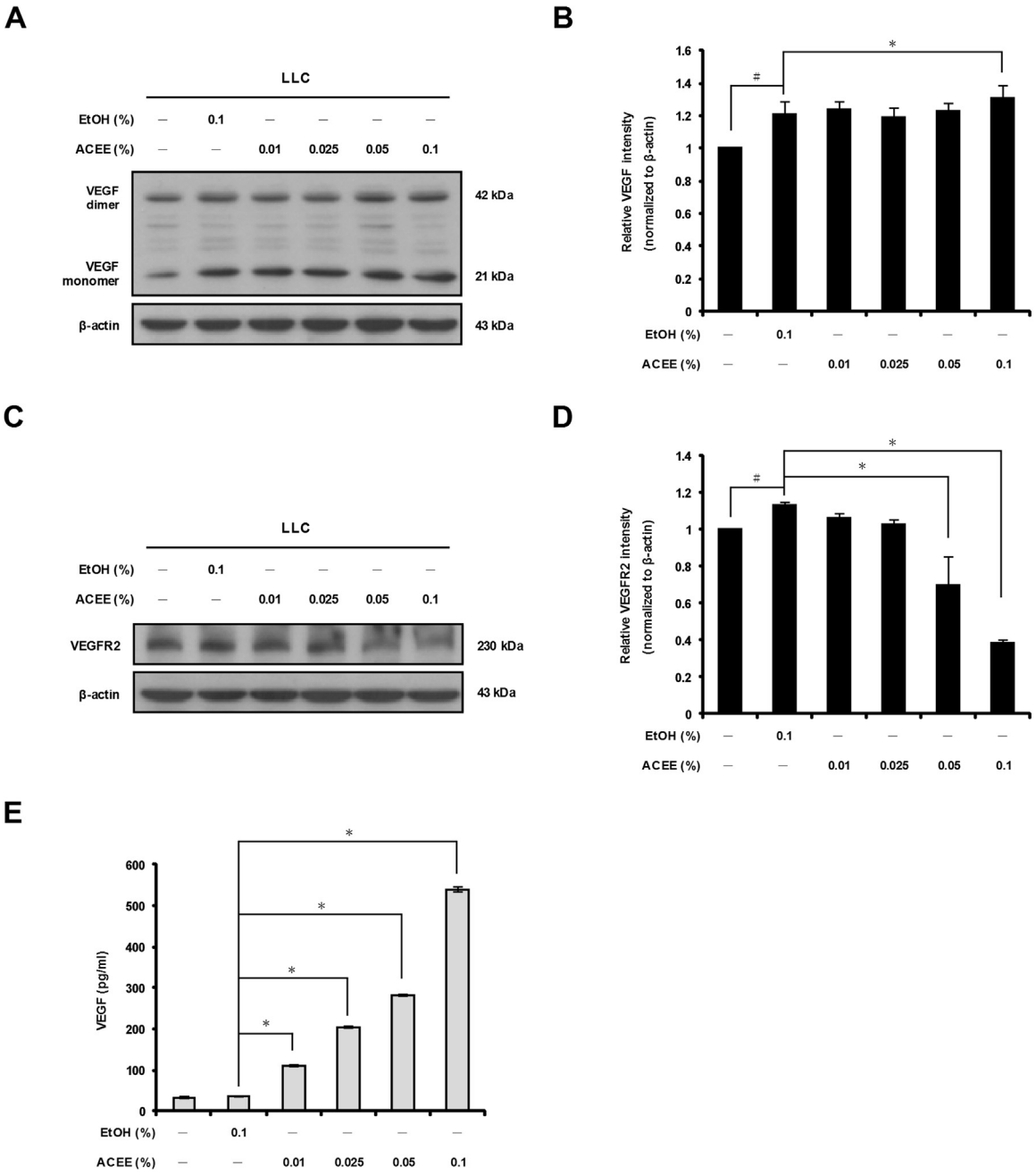

Fig. 6. ACEE suppresses VEGFR2 protein expression in LLC cells. (A) Cells were exposed to ACEE at the indicated concentrations for 6 h, and VEGF expression level was monitored by Western blot analysis. β-actin was used as an internal control. (B) VEGF band intensity was quantified by densitometry and normalized to β-actin. (C) VEGFR2 protein expression was examined using Western blot analysis. (D) Relative VEGFR2 band density quantified by densitometry and normalized to β-actin. (E) Secreted VEGF protein level in cell culture supernatants was monitored by ELISA. Data are presented as means ± SEM of three experiments performed in duplicate. $^{\#}P < 0.05$ versus untreated cells. $^{*}P < 0.05$ versus control ethanol-treated cells.

3.6. ACEE inhibits angiogenesis in allograft tumor tissues of mice

We performed immunohistochemistry analysis to examine the effects of ACEE on the expression of CD31, VEGF and VEGFR2 (angiogenesis markers) in allograft lung metastases produced by injection of LLC cells into the right hind paw of mice. As shown in Fig. 7A and B, expression of CD31, an indicator of microvessel density (MVD) used to quantify angiogenesis, was downregulated in ACEE-treated mice compared with the control group (Day 2 and 15). In addition, treatment with ACEE led to a decrease of VEGFR2 expression, while VEGF was not affected (Fig. 7A, C and D). Collectively, these results indicate that ACEE inhibits angiogenesis *in vitro* and *in vivo* by preventing activation of the VEGFR2-STAT3 signaling pathway.

4. Discussion

The observation that angiogenesis plays a pivotal role in tumor growth and metastasis has led to the development of anti-angiogenesis therapy for the treatment of solid tumors (Fontanella et al., 2014). Nonetheless, novel treatments that target cancer development and metastases are still highly needed. In the present study, we show that ACEE treatment inhibits proliferation, migration and capillary formation in endothelial cells, processes that represent critical steps during angiogenesis. ACEE also suppresses blood vessel formation in the Matrigel plug assay *in vivo*. Furthermore, immunohistochemistry analysis of lung tumor sections revealed that ACEE reduces expression of angiogenesis markers in metastatic lung tumors. Based on these findings,

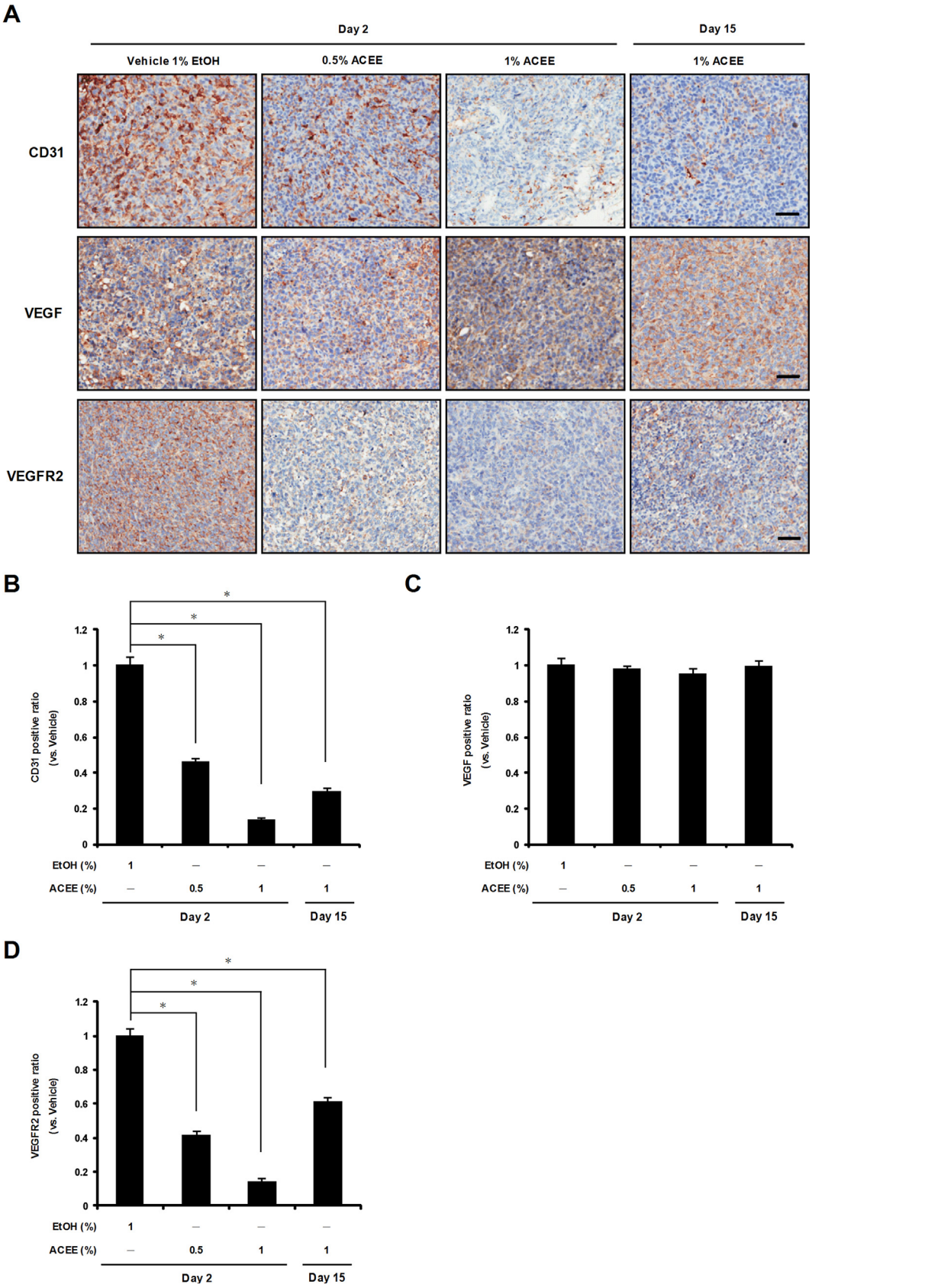

Fig. 7. ACEE inhibits tumor angiogenesis *in vivo*. LLC cells (2.5×10^5 cells) were inoculated into the right hind paw of 8-week-old male C57BL/6 mice (n = 5 mice/group) on day 1. The mice were treated with ethanol (1%) or ACEE (0.5% or 1%) from day 2 or day 15 by oral gavage five times per week. The mice were sacrificed on day 45 and lung tumor tissues were prepared for immunohistochemistry. (A) Representative images of paraffin-embedded lung tumor tissues stained for CD31, VEGF and VEGFR2 are shown. CD31 staining reflects tumor microvessel density, while VEGF and VEGFR2 staining shows angiogenesis levels. (B–D) Quantitative analysis of the staining shown in A. Data are presented as means ± SEM (n = 5/group). * $P < 0.05$ versus the vehicle group. Scale bar = 100 μm.

Table 1
Chemical analysis of ACEE.

Carbohydrates	2.5%
Lipids	91.4%
Saturated lipids	14.4%
Trans fats	Not detected
Triterpenoids	34.3%
Pinicolol B	91 ppm
Proteins	2.0%
Sodium	0.3%
Calories	841 (kcal/100 g)

Percentages represent weight/weight values (w/w, or g/100 g).
Abbreviations: ppm, parts per million; kcal, kilo-calories.

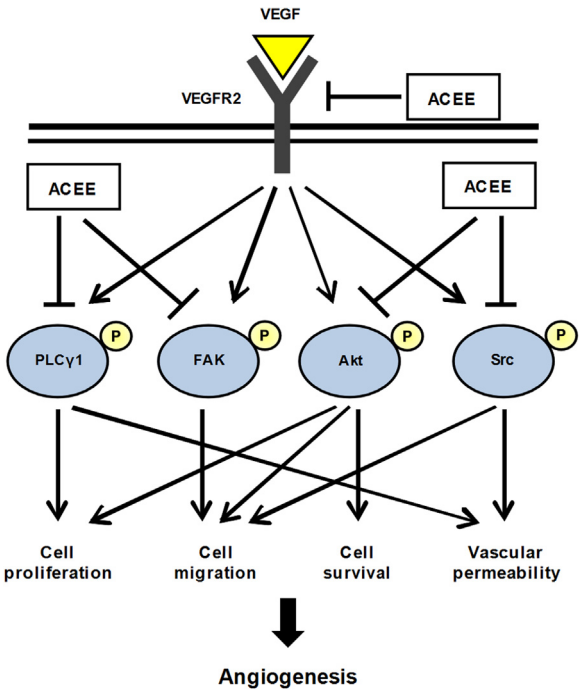

Fig. 8. Proposed model of the effects of ACEE on angiogenesis in endothelial cells. ACEE inhibits angiogenesis by blocking activation of VEGFR2 and downstream intracellular kinases in endothelial cells.

we conclude that ACEE may be used to inhibit angiogenesis as part of cancer treatment protocols.

Targeting of VEGFR2, which is involved in the process of tumor angiogenesis, has already been used in anti-cancer therapy (McMahon, 2000; Fontanella et al., 2014). Inhibitors of receptor tyrosine kinase, which prevent VEGFR2 phosphorylation, represent promising anti-angiogenic drugs that block intracellular signaling pathways involved in vascular endothelial cell proliferation, migration, survival and permeability (Holmes et al., 2007; Fontanella et al., 2014; Zhang et al., 2016). VEGFR2 signaling activates many downstream mediators, including JAK2 and STAT3, which affect cell proliferation, migration and capillary formation in endothelial cells (Dong et al., 2010). We observed that ACEE inhibits VEGFR2 phosphorylation and VEGFR2-mediated JAK2/STAT3 signaling in human endothelial cells. ACEE treatment dose-dependently reduces phosphorylation and activation of intracellular signaling kinases that act downstream of VEGFR2 in HUVECs, including PLCγ1, FAK, Src and Akt. *A. cinnamomea* also inhibits the JAK2/STAT3 signaling pathway in LLC cells (Huang et al., submitted for publication). These results suggest that ACEE may suppress angiogenesis at least in part by inhibiting the VEGFR2-JAK2-STAT3 signaling pathway.

Previous studies have shown that polysaccharides isolated from *A. cinnamomea* inhibit angiogenesis in endothelial cells (Cheng et al., 2005, 2011; Yang et al., 2009). On the other hand, the ACEE studied here contains mainly lipids (91.4%) and only a relatively small amount of carbohydrates (2.5%) (Table 1), an observation which suggests that compounds other than polysaccharides may be responsible for the anti-angiogenic effects reported in the present study. Triterpenoids (34.3%) and the lanostanoid compound pinicolol B (91 ppm) have been identified in ACEE (Table 1), and previous studies have shown that these compounds possess anti-cancer activities (Petronelli et al., 2009; Wu et al., 2017). Future studies are needed to determine the effects of these and other *A. cinnamomea*-derived compounds on angiogenesis and metastasis.

In conclusion, the present study reveals that an extract of *A. cinnamomea* mycelium effectively inhibits angiogenesis *in vitro* and *in vivo* (as illustrated in Fig. 8). ACEE exerts anti-angiogenic effects by down-regulating VEGFR2 phosphorylation and suppressing downstream signaling pathways that play an important role in regulating angiogenesis in endothelial cells. These findings suggest that at least part of the anti-cancer effects of ACEE reported earlier may be partially attributed to the anti-angiogenesis mechanism identified here. ACEE represents an interesting candidate for the development of novel anti-angiogenesis treatments and the isolation of active compounds to treat cancer and other angiogenesis-related diseases.

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Author contributions

- Conceived and designed the experiments: Tsung-Teng Huang, Yun-Fei Ko, Hsin-Chih Lai, John D. Young and Kowit-Yu Chong.
- Performed the experiments: Tsung-Teng Huang and Ying-Wei Lan.
- Analyzed the data: Tsung-Teng Huang, Ying-Wei Lan, Yun-Fei Ko, Chuan-Mu Chen, Hsin-Chih Lai, David M. Ojcius, Jan Martel, John D. Young and Kowit-Yu Chong.
- Wrote the manuscript: Tsung-Teng Huang, David M. Ojcius, Jan Martel, John D. Young and Kowit-Yu Chong.

Conflict of interest

Y.-F.K. is President of Chang Gung Biotechnology Corporation. J.D.Y. is Chairman of the Board of Chang Gung Biotechnology Corporation. The authors own patents related to the preparation and use of medicinal mushrooms and derived compounds.

References

Ao, Z.H., Xu, Z.H., Lu, Z.M., Xu, H.Y., Zhang, X.M., Dou, W.F., 2009. Niuchangchih (*Antrodia camphorata*) and its potential in treating liver diseases. J. Ethnopharmacol. 121, 194–212.

Chen, K.C., Peng, C.C., Peng, R.Y., Su, C.H., Chiang, H.S., Yan, J.H., Hsieh-Li, H.M., 2007. Unique formosan mushroom *Antrodia camphorata* differentially inhibits androgen-responsive LNCaP and -independent PC-3 prostate cancer cells. Nutr. Cancer 57, 111–121.

Chen, Y.J., Cheng, P.C., Lin, C.N., Liao, H.F., Chen, Y.Y., Chen, C.C., Lee, K.M., 2008. Polysaccharides from *Antrodia camphorata* mycelia extracts possess immunomodulatory activity and inhibits infection of *Schistosoma mansoni*. Int. Immunopharmacol. 8, 458–467.

Chang, C.J., Lin, C.S., Lu, C.C., Martel, J., Ko, Y.F., Ojcius, D.M., Tseng, S.F., Wu, T.R., Chen, Y.Y.M., Young, J.D., Lai, H.C., 2015. *Ganoderma lucidum* reduces obesity in mice by modulating the composition of the gut microbiota. Nat. Commun. 6, 7489.

Cheng, J.J., Huang, N.K., Chang, T.T., Wang, D.L., Lu, M.K., 2005. Study for anti-angiogenic activities of polysaccharides isolated from *Antrodia cinnamomea* in endothelial cells. Life Sci. 76, 3029–3042.

Cheng, J.J., Lu, M.K., Lin, C.Y., Chang, C.C., 2011. Characterization and functional elucidation of a fucosylated 1,6-α-D-mannogalactan polysaccharide from *Antrodia cinnamomea*. Carbohydr. Polym. 83, 545–553.

Chiang, P.C., Lin, S.C., Pan, S.L., Kuo, C.H., Tsai, I.L., Kuo, M.T., Wen, W.C., Chen, P., Guh, J.H., 2010. Antroquinonol displays anticancer potential against human hepatocellular carcinoma cells: a crucial role of AMPK and mTOR pathways. Biochem. Pharmacol. 79, 162–171.

Dong, Y., Lu, B., Zhang, X., Zhang, J., Lai, L., Li, D., Wu, Y., Song, Y., Luo, J., Pang, X., Yi, Z., Liu, M., 2010. Cucurbitacin E, a tetracyclic triterpenes compound from Chinese medicine, inhibits angiogenesis through VEGFR2-mediated Jak2–STAT3 signaling pathway. Carcinogenesis 31, 2097–2104.

Fan, J.P., He, C.H., 2006. Simultaneous quantification of three major bioactive triterpene acids in the leaves of *Dyospyros kaki* by high-performance liquid chromatography method. J. Pharm. Biomed. Anal. 41, 950–956.

Ferrara, N., Kerbel, R.S., 2005. Angiogenesis as a therapeutic target. Nature 438, 967–974.

Fontanella, N., Ongaro, E., Bolzonello, S., Guardascione, M., Fasola, G., Aprile, G., 2014. Clinical advances in the development of novel VEGFR2 inhibitors. Ann. Transl. Med. 2, 123.

Geethangili, M., Tzeng, T.M., 2011. Review of pharmacological effects of *Antrodia camphorata* and its bioactive compounds. Evid.-Based Complement. Altern. Med. 2011, 212641.

Holmes, K., Roberts, O.L., Thomas, A.M., Cross, M.J., 2007. Vascular endothelial growth factor receptor-2: structure, function, intracellular signalling and therapeutic inhibition. Cell. Signal. 19, 2003–2012.

Holmes, D., 2015. Obesity: medicinal mushroom reduces obesity by modulating microbiota. Nat. Rev. Endocrinol. 11, 504.

Huang, T.T., Wu, S.P., Chong, K.Y., Ojcius, D.M., Ko, Y.F., Wu, Y.H., Wu, C.Y., Lu, C.C., Martel, J., Young, J.D., Lai, H.C., 2014. The medicinal fungus *Antrodia cinnamomea* suppresses inflammation by inhibiting the NLRP3 inflammasome. J. Ethnopharmacol. 155, 154–164.

Huang, T.T., Lai, H.C., Ko, Y.F., Ojcius, D.M., Lan, Y.W., Martel, J., Young, J.D., Chong, K.Y., 2015. *Hirsutella sinensis* mycelium attenuates bleomycin-induced pulmonary inflammation and fibrosis *in vivo*. Sci. Rep. 5, 15282.

Huang, T.T., Lan, Y.W., Chen, C.M., Ko, Y.F., Lai, H.C., Ojcius, D.M., Martel, J., Young, J. D., Chong, K.Y., 2018. *Antrodia cinnamomea* induces anti-tumor activity by inhibiting the STAT3 signaling pathway in lung cancer cells (submitted for publication).

Kerbel, R., Folkman, J., 2002. Clinical translation of angiogenesis inhibitors. Nat. Rev. Cancer 2, 727–739.

Kim, M.J., Nam, H.J., Kim, H.P., Han, S.W., Im, S.A., Kim, T.Y., Oh, D.Y., Bang, Y.J., 2013. OPB-31121, a novel small molecular inhibitor, disrupts the JAK2/STAT3 pathway and exhibits an antitumor activity in gastric cancer cells. Cancer Lett. 335, 145–152.

Liu, L., Nam, S., Tian, Y., Yang, F., Wu, J., Wang, Y., Scuto, A., Polychronopoulos, P., Magiatis, P., Skaltsounis, L., Jove, R., 2011. Polychronopoulos P, Magiatis P, Skaltsounis L, Jove R. 6-Bromoindirubin-3′-oxime inhibits JAK/STAT3 signaling and induces apoptosis of human melanoma cells. Cancer Res. 71, 3972–3979.

Martel, J., Ojcius, D.M., Chang, C.J., Lin, C.S., Lu, C.C., Ko, Y.F., Tseng, S.F., Lai, H.C., Young, J.D., 2017a. Anti-obesogenic and antidiabetic effects of plants and mushrooms. Nat. Rev. Endocrinol. 13, 149–160.

Martel, J., Ko, Y.F., Ojcius, D.M., Lu, C.C., Chang, C.J., Lin, C.S., Lai, H.C., Young, J.D., 2017b. Immunomodulatory properties of plants and mushrooms. Trends Pharmacol. Sci. 38, 967–981.

Martel, J., Ko, Y.F., Liau, J.C., Lee, C.S., Ojcius, D.M., Lai, H.C., Young, J.D., 2017c. Myths and realities surrounding the mysterious caterpillar fungus. Trends Biotechnol. 35, 1017–1021.

McMahon, G., 2000. VEGF receptor signaling in tumor angiogenesis. Oncologist 5, 3–10.

Olsson, A.K., Dimberg, A., Kreuger, J., Claesson-Welsh, L., 2006. VEGF receptor signaling — in control of vascular function. Nat. Rev. Mol. Cell Biol. 7, 359–371.

Patan, S., 2004. Vasculogenesis and angiogenesis. Cancer Treat. Res. 117, 3–32.

Peng, C.C., Chen, K.C., Peng, R.Y., Chyau, C.C., Su, C.H., Hsieh-Li, H.M., 2007. *Antrodia camphorata* extract induces replicative senescence in superficial TCC, and inhibits the absolute migration capability in invasive bladder carcinoma cells. J. Ethnopharmacol. 109, 93–103.

Petronelli, A., Pannitteri, G., Testa, U., 2009. Triterpenoids as new promising anticancer drugs. Anticancer Drugs 20, 880–892.

Song, T.Y., Yen, G.C., 2002. Antioxidant properties of *Antrodia camphorata* in submerged culture. J. Agric. Food Chem. 50, 3322–3327.

Wasser, S.P., 2014. Medicinal mushroom science: current perspectives, advances, evidences, and challenges. Biomed. J. 37, 345–356.

Wu, T.R., Huang, T.T., Martel, J., Liau, J.C., Chiu, C.Y., Leu, Y.L., Jian, W.T., Chang, I.T., Lu, C.C., Ojcius, D.M., Ko, Y.F., Lai, H.C., Young, J.D., 2017. Pinicolol B from *Antrodia cinnamomea* induces apoptosis of nasopharyngeal carcinoma cells. J. Ethnopharmacol. 201, 117–122.

Yang, H.L., Chen, C.S., Chang, W.H., Lu, F.J., Lai, Y.C., Chen, C.C., Hseu, T.H., Kuo, C.T., Hseu, Y.C., 2006. Growth inhibition and induction of apoptosis in MCF-7 breast cancer cells by *Antrodia camphorata*. Cancer Lett. 231, 215–227.

Yang, C.M., Zhou, Y.J., Wang, R.J., Hu, M.L., 2009. Anti-angiogenic effects and mechanisms of polysaccharides from *Antrodia cinnamomea* with different molecular weights. J. Ethnopharmacol. 123, 407–412.

Yuan, G., Yan, S., Xue, H., Zhang, P., Sun, J., Li, G., 2015. JSI-124 suppresses invasion and angiogenesis of glioblastoma cells in vitro. PLoS One 10, e0118894.

Zhang, J., Liu, C., Shi, W., Yang, L., Zhang, Q., Cui, J., Fang, Y., Li, Y., Ren, G., Yang, S., Xiang, R., 2016. The novel VEGF receptor 2 inhibitor YLL545 inhibits angiogenesis and growth in breast cancer. Oncotarget 7, 41067–41080.

Zhao, M., Gao, F.H., Wang, J.Y., Liu, F., Yuan, H.H., Zhang, W.Y., Jiang, B., 2011. JAK2/STAT3 signaling pathway activation mediates tumor angiogenesis by upregulation of VEGF and bFGF in non-small-cell lung cancer. Lung Cancer 73, 366–374.