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# Inhibition of STAT3 signaling and induction of SHP1 mediate antiangiogenic and antitumor activities of ergosterol peroxide in U266 multiple myeloma cells

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## Abstract

**Background:** Ergosterol peroxide (EP) derived from edible mushroom has been shown to exert anti-tumor activity in several cancer cells. In the present study, anti-angiogenic activity of EP was investigated with the underlying molecular mechanisms in human multiple myeloma U266 cells.

**Results:** Despite weak cytotoxicity against U266 cells, EP suppressed phosphorylation, DNA binding activity and nuclear translocation of signal transducer and activator of transcription 3 (STAT3) in U266 cells at nontoxic concentrations. Also, EP inhibited phosphorylation of the upstream kinases Janus kinase 2 (JAK2) and Src in a time-dependent manner. Furthermore, EP increased the expression of protein tyrosine phosphatase SHP-1 at protein and mRNA levels, and conversely silencing of the SHP-1 gene clearly blocked EP-mediated STAT3 inactivation. In addition, EP significantly decreased vascular endothelial growth factor (VEGF), one of STAT3 target genes at cellular and protein levels as well as disrupted *in vitro* tube formation assay. Moreover, EP significantly suppressed the growth of U266 cells inoculated in female BALB/c athymic nude mice and immunohistochemistry revealed that EP effectively reduced the expression of STAT3 and CD34 in tumor sections compared to untreated control.

**Conclusion:** These findings suggest that EP can exert antitumor activity in multiple myeloma U266 cells partly with antiangiogenic activity targeting JAK2/STAT3 signaling pathway as a potent cancer preventive agent for treatment of multiple myeloma cells.

**Keywords:** ergosterol peroxide, JAK2, STAT3, angiogenesis, multiple myeloma

## Background

Ergosterol Peroxide (EP), 5 $\alpha$ , 8 $\alpha$ -epidioxy-22E-ergosta-6, 22-dien-3 $\beta$ -ol, is found in plants [1], lichens [2] and mushrooms such as *Ganoderma lucidum* [3], *Sporothrix schenckii* [4] and *Cordyceps sinensis* [5]. Despite various biological effects of EP such as immunosuppressive [6-8], anti-viral [9], anti-inflammatory [10] and anti-tumor [5,10] activities, the underlying molecular

mechanisms for anti-cancer activity of EP still remain unclear.

STAT proteins originally discovered as latent cytoplasmic transcription factors [11] are involved in a variety of cellular processes such as cell proliferation, differentiation and apoptosis [12,13]. Of the STAT proteins, STAT3 is often constitutively activated in many human cancer cells including multiple myeloma, leukemia, lymphoma, and solid tumors [14,15]. Upon activation, STAT3 undergoes phosphorylation-induced homodimerization, leading to nuclear translocation, DNA binding and subsequent gene transcription. STAT3 phosphorylation is mediated through the activation of

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non-receptor protein tyrosine kinases Janus activated kinases (JAKs) and c-Src [16].

STAT3 participates in oncogenesis through up-regulation of genes encoding apoptosis inhibitors such as bcl-x<sub>L</sub>, bcl-2, and survivin [15]. Also, recent studies reported the evidences that STAT3 is involved in the regulation of angiogenesis through modulation of VEGF, a key regulator of angiogenesis [17-19]. In this regards, we investigated anti-angiogenic effect of EP in association with JAK2/STAT3 pathway and STAT3 related VEGF expression in U266 cells *in vitro* and mouse xenograft model.

## Methods

### Isolation of ergosterol peroxide (EP)

EP was isolated according to the Krzyczkowski's method as previously described [20].

### Cell culture

U266 (multiple myeloma), SCC4 (head and neck squamous cell carcinoma), DU145 (prostate cancer), and MDA-MB-231 (breast cancer) cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD) and maintained in RPMI 1640 supplemented with 10% FBS and antibiotics. Human umbilical vein endothelial cells (HUVECs) were isolated from fresh human umbilical cord vein by collagenase treatment as described previously [21]. The cells were maintained in in EBM-2 containing 2% FBS, 0.04% hydrocortisone, 0.4% hFGF-B, 0.1% VEGF, 0.1% IGF-1, 0.1% ascorbic acid, 0.1% hEGF, 1% GA-1000, and 1% Heparin (Walkersville, MD)

### Cytotoxicity assay

The cytotoxic effect of EP was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. U266 cells were seeded onto 96-well microplate at a density of  $1 \times 10^4$  cells/well and treated with various concentrations of EP (0, 6.25, 12.5, 25 or 50  $\mu$ M) for 24 h. The culture medium was removed after centrifuging the plate and MTT solution (1 mg/ml) was added to each well until formazan was constituted. MTT lysis solution (20% SDS and 50% dimethylformamide) was added to dissolve formazan and optical density (OD) was measured using microplate reader (Tecan Austria GmbH, Grödig, Austria) at 570 nm. Cell viability was calculated as a percentage of viable cells in EP-treated group versus untreated control by following equation.

$$\text{Cell viability (\%)} = \frac{[\text{OD (EP)} - \text{OD (Blank)}]}{[\text{OD (Control)} - \text{OD (Blank)}]} \times 100$$

### Western blotting

Cells lysates were prepared using lysis buffer [50 mM Tris (pH 7.4), 300 mM NaCl, 5 mM EDTA (pH 8.0),

0.5% Triton X-100, 1 mM aprotinin, 1 mM leupeptin, 1 mM pepstatin, 10 mM iodoacetamide, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM NaVO<sub>4</sub>] and centrifuged at  $14,000 \times g$  for 10 min at 4°C. Protein samples were collected, separated onto 10-12% SDS-PAGE gels and electrotransferred to a nitrocellulose membrane. The membranes were blocked in 5% nonfat skim milk, probed with primary antibodies for STAT3, phospho-STAT3<sup>Y705</sup>, JAK2, phospho-JAK2<sup>Y1007/1008</sup>, Src, phospho-Src<sup>Y416</sup> (Cell Signaling, Danvers, MA), SHP-1, VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) and  $\beta$ -actin (Sigma, St. Louis, MO) at 4°C for overnight, and then exposed to HRP-conjugated secondary antibodies at room temperature for 2 h. Protein expression was detected by using enhanced chemiluminescence (ECL) kit (Amersham Pharmacia, Piscataway, NJ).

### Electrophoretic mobility shift assay (EMSA) for STAT3-DNA binding

The STAT3-DNA binding was analyzed by electrophoretic mobility shift assay (EMSA) using a <sup>32</sup>P-labeled high-affinity sis-inducible element (hSIE) probe (5'-CTTCATTTCCCGTAAATCCCTAAAGCT-3' and 5'-AGCTTTAGGGATTTACGG GAAA TGA-3') as previously described [22]. Briefly, nuclear extracts were incubated with the hSIE probe and the protein-DNA complexes were separated onto 5% native polyacrylamide gels. The gels were dried, and the radioactive bands were quantitated with Storm 820 and Imagequant software (Amersham Pharmacia, Piscataway, NJ).

### RT-PCR analysis

Total RNA was extracted by using Trizol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 1  $\mu$ g of total RNA and subjected to PCR reaction by using Superscript One Step reverse transcription-PCR (RT-PCR) kit (Invitrogen). The PCR conditions were 30 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min. The primer sequences were as follows: *SHP-1* (forward primer 5'-AAT GCG TCC CAT ACT GGC CCG A-3'; reverse primer 5'-CCC GCA GTT GGT CAC AGA GT-3'), and *GAPDH* (forward primer 5'-TCA CCA TCT TCC AGG AGC GA-3'; reverse primer 5'-CAC AAT GCC GAA GTG GTG GT-3').

### siRNA transfection

siRNA oligonucleotides for SHP-1 (SantaCruz biotechnology, SantaCruz, CA) were transfected by using LipofectAMINE transfection reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocols.

### Enzyme-linked immunosorbent assay (ELISA) for VEGF

U266 cells were seeded onto 6-well plates at a density of  $1 \times 10^6$  cells/well and treated with 25  $\mu$ M EP. The

VEGF levels in the supernatant were measured by using a Quantikine VEGF ELISA kit (R&D systems, Minneapolis, MN) according to manufacturer's protocols.

#### **In vitro tube formation assay**

Tube formation assay was performed on Matrigel (Becton Dickinson Labware, Bedford, MA) as described previously [23]. In brief, human umbilical vein endothelial cells (HUVECs) ( $3 \times 10^4$  cells/well) were seeded onto Matrigel-coated 24-well plates and treated with VEGF (20 ng/ml) in the absence or presence of EP (0, 10 or 25  $\mu$ M) for 6 h. Cells were fixed with 2% paraformaldehyde and stained with 2% crystal violet. Tube formation was observed under an Axiovert S 100 light microscope (Carl Zeiss, Weimar, Germany) and counted in randomly selected areas using NIH Scion image program.

#### **Mouse xenograft model**

Six-week-old female athymic nude mice were purchased from Jung Ang lab animal (Seoul, Republic of Korea) and maintained under conventional conditions. U266 cells ( $2 \times 10^6$  cells) were mixed with Matrigel (Becton Dickinson, 50%, in 100  $\mu$ l) and injected subcutaneously on the right flank of the mice. After 5 days of inoculation, the mice ( $n = 5$ /group) were given intraperitoneal (i.p) injection of EP at 100 mg/Kg in 2% tween-80, or bortezomib *i.p.* 0.25 mg/kg in PBS every 2 or 3 days. Control mice were administered the solvent vehicle. Tumor volume was measured every other day with caliper and calculated according to the formula;  $V = 0.25a^2b$ , where  $a$  is the smallest superficial diameter and  $b$  is the largest superficial diameter.

#### **Immunohistochemistry**

The animals were sacrificed 20 days after U266 inoculation, and tumors were immediately removed, fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned at 4  $\mu$ m. Immunohistochemical staining for phospho-STAT3(Cell Signaling Technology, Danvers, MA), CD 34 (microvessel marker, Abcam, Boston, MA) and TUNEL (Calbiochem, Darmstadt, Germany) was performed and detected by DAB substrate staining (brown).

#### **Statistical analysis**

All values were expressed as means  $\pm$  S.D. Data were analyzed by one-way analysis of variance (ANOVA) and by student's t-test (Sigma plot<sup>®</sup>, San Rafael, CA, USA).

## **Results**

### **Ergosterol peroxide suppresses STAT3 activation in U266 cells**

STAT3 is implicated in cell survival and growth in multiple myeloma cells [14]. In light of this event, we examined whether EP can modulate the constitutive

activation of STAT3 in U266 cells. EP significantly inhibited the constitutive activation of STAT3 in time- and dose-dependent manners (Figure 1A and 1B, respectively). Tyrosine phosphorylation of STATs causes their dimerization and translocation to the nucleus, where they bind to DNA and regulate gene transcription [22]. Furthermore, we evaluated whether EP can affect on constitutive STAT3 activation in other cancer cell lines. SCC4 (head and neck squamous cell carcinoma), DU145 (prostate cancer), and MDA-MB-231 (breast cancer) cells expressing constitutive STAT3 were treated with or without EP. As shown in Figure 1C, EP inhibited STAT3 activation without affecting total STAT3 level. Consistent with the results of Western blotting in Figure 1A and 1B, gel shift assay showed that EP clearly inhibited the STAT3 DNA-binding activity in time- and dose-dependent manners (Figure 1D and 1E, respectively).

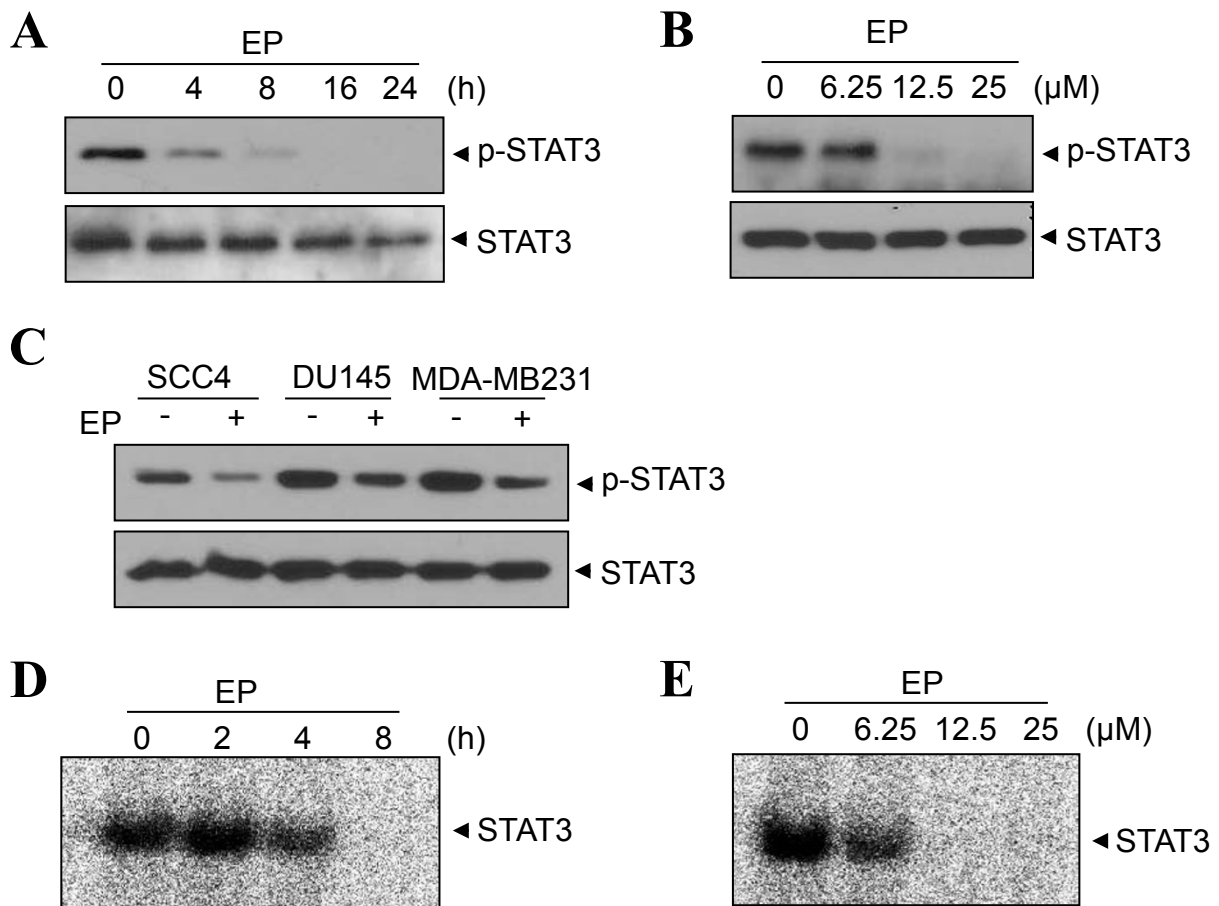
### **Ergosterol peroxide suppresses JAK2 and Src in U266 cells**

STAT3 is activated by soluble tyrosine kinases JAKs [24]. Effect of EP was measured on the activation of JAK2 by Western blotting. EP suppressed the phosphorylation of JAK2 in a time-dependent manner (Figure 2A), while it did not change the level of JAK1 (data not shown). The Src is another tyrosine kinase family to activate STAT3 signaling [16]. EP also decreased the phosphorylation of Src in a time-dependent manner (Figure 2B). These results indicate that EP inactivates the upstream kinases JAK2 and Src of STAT3 in U266 cells.

### **Ergosterol peroxide enhances expression of SHP-1 in U266 cells**

Non-transmembrane protein tyrosine phosphatases (PTPs) play important roles in the negative regulation of the JAK/STAT signaling [25]. Thus, we investigated whether PTPs are involved in EP-mediated STAT3 inactivation using a PTP inhibitor sodium pervanadate. As shown in Figure 3A, pervanadate reversed EP-induced STAT3 inactivation in a dose-dependent manner, indicating the important role of PTP(s) in EP induced STAT3 inactivation in U266 cells.

Since SHP-1 is a PTP expressed most abundantly in hematopoietic cells [26,27], the effect of EP on the expression of SHP-1 was examined. EP enhanced SHP-1 protein expression in time- and dose-dependent manners (Figure 3B and 3C). Consistently, EP increased the expression of SHP-1 at the mRNA level in a time-dependent manner (Figure 3D). Conversely, EP-mediated SHP-1 was reduced in a time course by the removal of EP containing media, indicating that SHP-1 expression by EP is reversible (Figure 3E). Furthermore,



**Figure 1 Ergosterol peroxide (EP) suppresses STAT3 activation in U266 cells.** (A) Cells were treated with 25  $\mu$ M EP for 0, 4, 8, 16 or 24 h. (B) Cells were treated with various concentrations of EP (0, 6.25, 12.5 or 25  $\mu$ M) for 8 h. (C) SCC4, DU145 and MDA-MB-231 cells were treated with or without EP (25  $\mu$ M) for 8 h. Whole cell extracts were prepared and subjected to Western blotting to determine level of phospho-STAT3 and STAT3. (D and E) Gel shift mobility assay was performed using nuclear extracts to examine the STAT3-DNA binding activity.

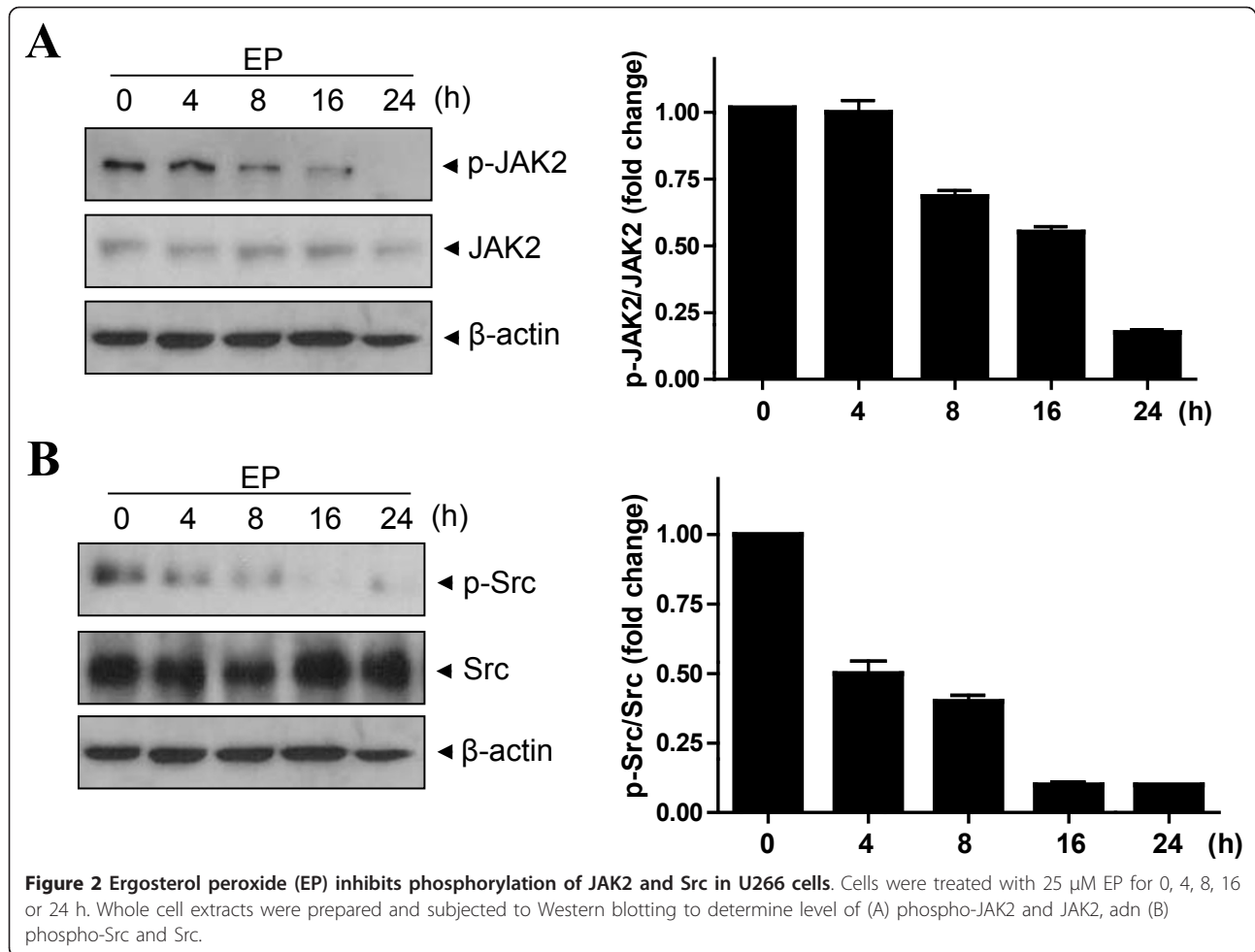
blocking SHP-1 using its specific siRNA clearly abolishes the ability of EP to inhibit STAT3 while scrambled siRNA had no effect on the expression (Figure 3F). Taken together, these results showed evidence that SHP-1 plays a critical role in the suppression of STAT3 phosphorylation by EP.

#### Ergosterol peroxide (EP) exerts anti-angiogenic activity in vitro

Cytotoxicity of EP against U266 cells were determined by MTT assay. U266 cells were treated with various concentrations of EP (0, 6.25, 12.5, 25 or 50  $\mu$ M) for 24 h. As shown in Figure 4A, EP had no significant cytotoxic effect in U266 cells. STAT3 contributes to angiogenic regulation by inducing VEGF expression [28,29]. Thus, VEGF protein expression was analyzed at the cellular and protein levels in EP-treated cells. EP suppressed protein expression of VEGF in a time-dependent manner by Western blotting (Figure 4B).

Consistently, secretion of VEGF was significantly inhibited by EP treatment in time- and dose-dependent manners by ELISA (Figure 4C and 4D, respectively).

To further confirm the anti-angiogenic activity of EP, *in vitro* tube formation assay was conducted using HUVECs treated either with or without VEGF in the absence or presence of EP. As shown in Figure 4E, no tube formation was observed in untreated cells, while clear tube formation was exhibited in VEGF-treated control. Notably, EP treatment inhibited VEGF-induced tube formation in a dose-dependent manner. Then, we tested whether EP can affect VEGF-mediated STAT3 activation in HUVECs. As shown in Figure 4F, VEGF treatment clearly enhanced phosphorylation of STAT3 in HUVECs, which was supported by Ebrahim's evidences [30]. In contrast, total STAT3 levels were not significantly changed by the treatment of VEGF and/or EP (Figure 4F).



### Ergosterol peroxide exerts anti-tumor activity in vivo

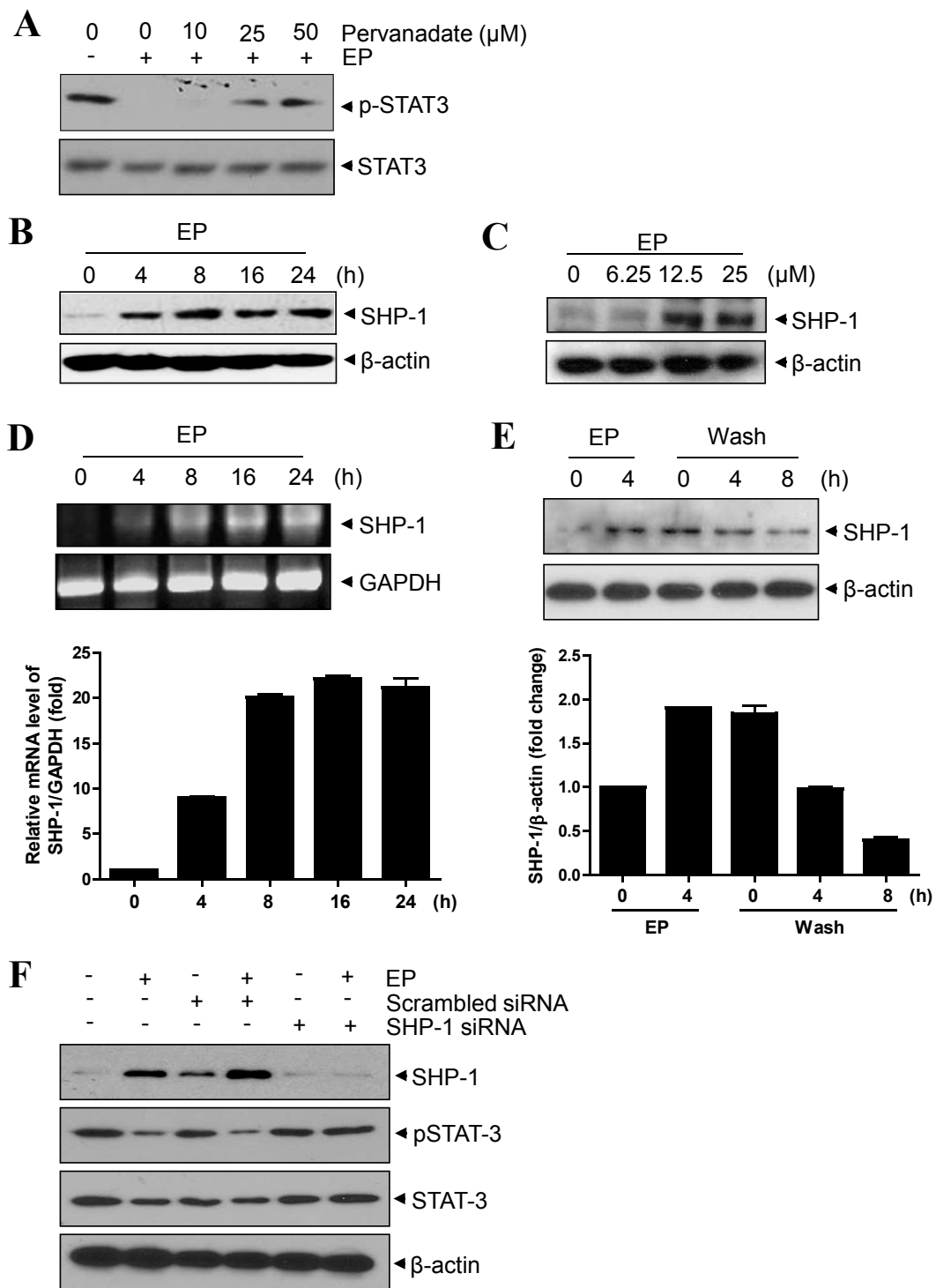
To confirm the anti-tumor efficacy of EP, U266 cells were subcutaneously inoculated into BALB/c athymic nude mice in the flank area, and starting after 5 days injection, the mice were administered EP or bortezomib (a positive control) every 2-3 day with intraperitoneal (*i. p.*) injection. Tumor growth was monitored every other day for 20 days. Tumor volumes in EP- or bortezomib-treated groups were decreased compared with untreated control (Figure 5A) without significant body weight loss in EP-treated group (Figure 5B).

Consistently, immunohistochemistry showed that EP treatment reduced the expression of phospho-STAT3 and angiogenesis marker CD34 compared to untreated control (Figure 5C), implying the antitumor effect of EP is associated with regulation of angiogenesis and STAT3 *in vitro* and *in vivo*. In addition, TUNEL positive cells for apoptosis were effectively increased in EP-treated tumor sections. However, considering that EP treatment for 48 h or 72 h exerted significant cytotoxicity against U266 cells, we can postulate that EP

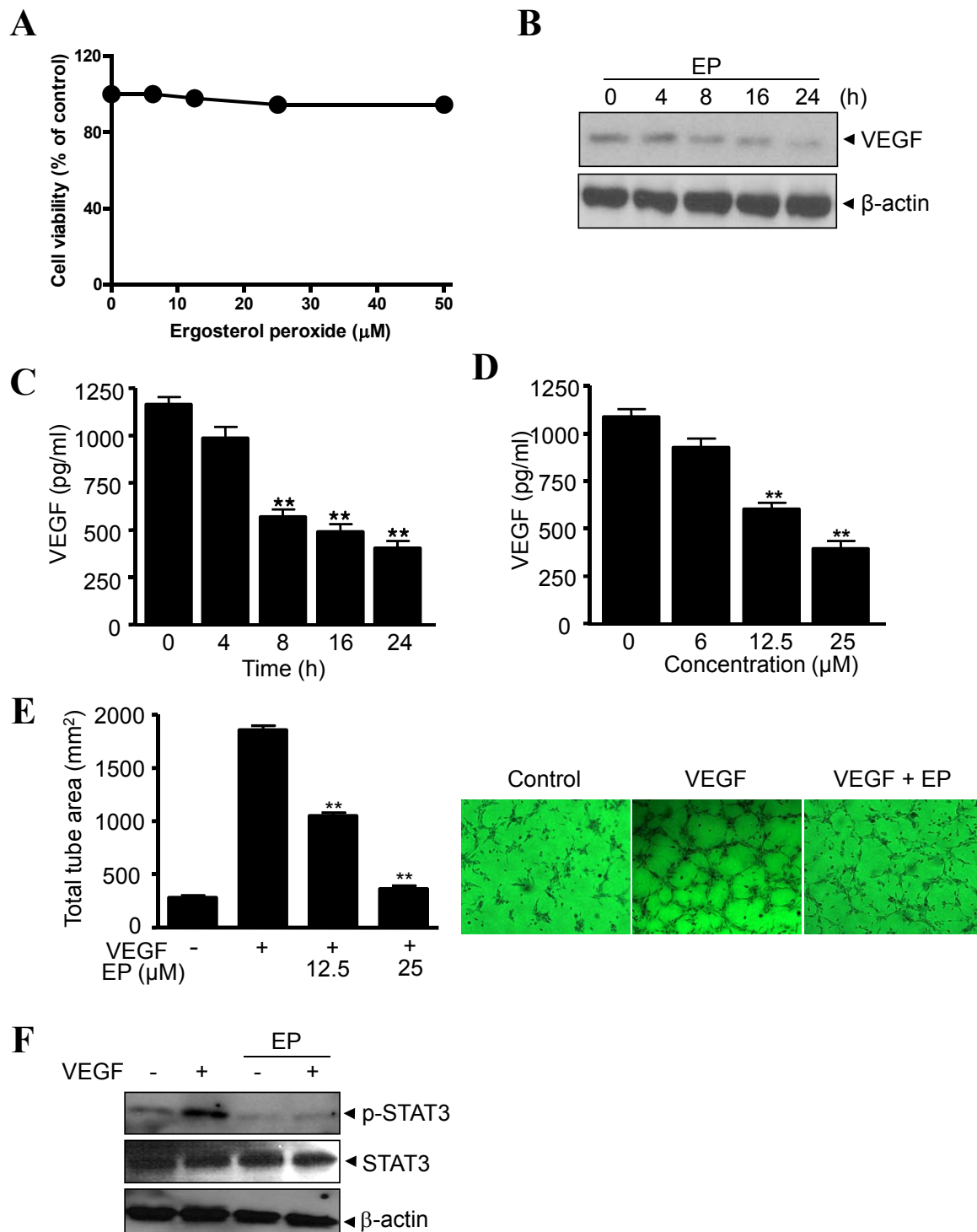
can exert antitumor effects via antiangiogenic activity at low concentration and apoptotic activity at higher concentration.

### Discussion

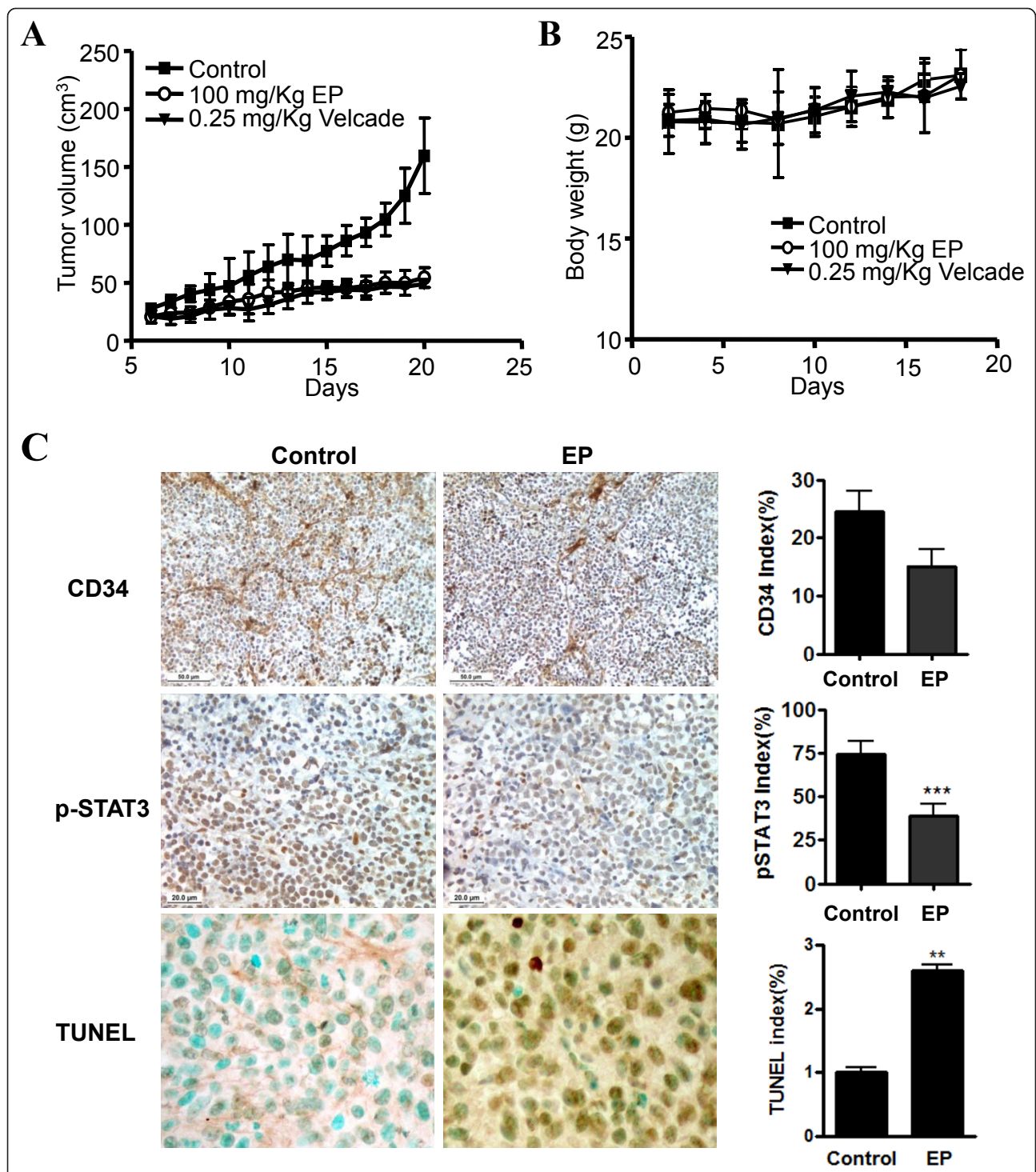
Multiple myeloma, also called plasma cells myeloma, is characterized by accumulation of secretory plasma cells. For the treatment of multiple myeloma, various chemotherapeutic agents such as vincristine, carmustine, mephalan, cyclophosphamide, doxorubicin, prednisone and dexamethasone have been used in combination or either drug alone [31]. However, many multiple myeloma patients frequently had no response to these agents and their prolonged exposure induced toxicity even in normal cells. Recently, phytochemicals are considerably advocated as rich sources of anti-cancer agents that deserve more rigorous and valuable investigations. Regarding this issue, several studies suggested phytochemicals such as curcumin [14], resveratrol [32] and capsaicin [33] as potent anti-cancer agents for multiple myeloma treatment. We also found that genipin [34]



**Figure 3 Ergosterol peroxide (EP) enhances expression of SHP-1 in U266 cells.** (A) Cells were treated with 25  $\mu$ M EP in the absence or presence of pervanadate (10, 25 or 50  $\mu$ M) for 4 h. Whole cell extracts were prepared and subjected to Western blotting to determine level of phospho-STAT3 and STAT3. (B and C) Cells were treated with 25  $\mu$ M EP for 0, 4, 8, 16 or 24 h (B) and various concentrations of EP (0, 6.25, 12.5 or 25  $\mu$ M) for 4 h (C). Whole cell extracts were prepared and subjected to Western blotting to determine SHP-1 expression. (D) Cells were treated with 25  $\mu$ M EP for 0, 4, 8, 16 or 24 h. RT-PCR was performed to analyze mRNA expression of SHP-1. (E) Cells were treated with 25  $\mu$ M EP for 4 h (left), or treated with 25  $\mu$ M EP for 1 h followed by washing PBS to remove EP and resuspension in fresh medium (right). Western blotting was performed for SHP-1. (F) Cells were transiently transfected with either SHP-1 or scrambled siRNA (50 nM) for 48 h and then treated with 25  $\mu$ M EP for 4 h. Western blotting was performed for phospho-STAT3 and STAT3.



**Figure 4 Ergosterol peroxide (EP) exerts anti-angiogenic activity *in vitro*.** (A) U266 cells were treated with various concentrations of EP (0, 6.25, 12.5, 25 or 50 μM) for 24 h. Cytotoxic effect of EP was evaluated by MTT assay. (B) U266 cells were treated with 25 μM EP for 0, 4, 8, 16 or 24 h. Cell lysates were prepared and subjected to Western blotting for VEGF. (C and D) U266 cells were seeded at density of  $1 \times 10^6$  cells/well onto 6-well plates and treated with 25 μM EP for 0, 4, 8, 16 or 24 h (C) and various concentrations of EP (0, 6, 12.5 or 25 μM) for 24 h (D). Levels of VEGF in the supernatants were measured by using a Quantikine VEGF ELISA kit. (E) For *in vitro* tube formation assay, HUVECs ( $3 \times 10^4$  cells/well) were seeded onto Matrigel coated 24-well plates and treated with VEGF (20 ng/ml) in the absence or presence of EP (0, 12.5 or 25 μM) for 6 h. Cells were fixed with 2% paraformaldehyde, stained with 2% crystal violet, and the number of tube was randomly counted in selected areas (left). Representative photographs of tube formation in cells treated with VEGF (20 ng/ml) in the absence or presence of 25 μM EP (right). Each experiment was repeated three times and all data were expressed as means  $\pm$  S.D. \*\*,  $p < 0.01$  vs untreated control. (F) HUVECs were treated with VEGF (20 ng/ml) in the absence or presence of 25 μM EP. Western blotting was performed for phospho-STAT3 and STAT3.



**Figure 5 Ergosterol peroxide (EP) exerts anti-tumor activity *in vivo*.** Six-week-old female BALB/c athymic nude mice were subcutaneously injected in the flank area with  $2 \times 10^6$  cells of U266 in 100  $\mu$ l of matrigel mixed PBS. Five days after inoculation, mice (n = 5/group) were each given every 2-3 day intraperitoneal (i.p.) injection of EP at 100 mg/kg in 2% tween-80 or bortezomib at 0.25 mg/kg in PBS. Control mice were administered the solvent vehicle. Tumor volumes were measured every other day with caliper. (A) Body weight was measured every other day after administration of EP or bortezomib. (B) Tumor volume was calculated according to the formula  $V = 0.25a^2b$ , where  $a$  is the smallest superficial diameter and  $b$  is the largest superficial diameter. (C) Mice were killed on day 20 after cell inoculation, and tumors were immediately removed, fixed, embedded and sectioned at 4  $\mu$ m for immunostaining of biomarkers. Representative photomicrographs of apoptosis detected by immunostaining of CD34, phospho-STAT3 and TUNEL at  $\times 400$ , 200 and 100 of magnification, respectively. The sections were detected as DAB substrate staining (brown) and counterstained with Mayer's hematoxylin solution (right). Graphs show the CD34 index (angiogenesis), TUNEL index (apoptosis) and, pSTAT3 index in tumor sections. \*\* $p < 0.01$  and \*\*\* $p < 0.001$  compared with vehicle-treated control mice (left).



and icariside II [35] could be applied for multiple myeloma therapy by inducing apoptosis and targeting the signaling molecules such as STAT3.

The STAT proteins were identified for the last decade as latent cytoplasmic transcription factors in response to all cytokine driven signaling [15]. Seven mammalian STAT proteins such as STAT1, 2, 3, 4, 5a, 5b and 6 act as multifunctional mediators to regulate various cellular processes such as cell proliferation, differentiation, angiogenesis, and apoptosis [36]. Especially, STAT3 is constitutively activated in many human cancers, including prostate cancer [37], breast cancer [38], squamous cell carcinoma of the head and neck (SCCHN) [39], nasopharyngeal carcinoma and multiple myelomas [40]. Thus, STAT3 is considered as a valuable therapeutic target molecule for cancer treatment.

Ergosterol peroxide (EP) is a steroid derivative isolated from medicinal mushroom [41]. Several studies reported anti-cancer activity of EP in various types of cancer cells. For instance, Russo and colleagues reported that EP attenuated cell growth and induced apoptosis in human prostate cancer LNCaP and DU-145 cells [42]. Kobori and colleagues reported that EP suppressed inflammatory response in RAW264.7 macrophages and growth of HT29 colon adenocarcinoma cells [43]. Also, Chen and colleagues suggested that EP from the fermentation mycelia of *Ganoderma lucidum* cultivated in the medium exerted the cytotoxic effect against Hep 3B cells [44]. In the present study, we found that EP exerts anti-cancer activity through the inhibition of angiogenesis by targeting the STAT3 signaling pathway in U266 cells *in vitro* and *in vivo*.

EP suppressed constitutive activation of STAT3 in U266, SCC4, DU145 and MDA-MB-231 cells. EP also inhibited the STAT3-DNA binding activity and the nuclear translocation of STAT3, suggesting that EP can prevent STAT3 activation at the transcriptional level. The inhibitory effect of EP on STAT3 activation was partly associated with the inhibition of upstream kinases JAK2 and Src by EP treatment. Furthermore, protein tyrosine phosphatases (PTPs) are known to be implicated in STAT3 signaling, including SHP-1 [45], SHP-2 [46], PTEN [47], SOCS-1 [48] and so on. Our results revealed that EP remarkably enhanced the expression of SHP-1 at levels of protein and mRNA. In contrast, EP had no significant effect on other PTPs such as SHP-2 and PTEN (data not shown). To confirm the significance of SHP-1, we utilized PTP inhibitor pervanadate or SHP-1 siRNA to block the expression of SHP-1. As expected, EP failed to inhibit STAT3 activation in the presence of pervanadate or SHP-1 siRNA, supporting that SHP-1 plays a critical role in dephosphorylating STAT3 by EP in U266 cells.

Recently, Niu and colleagues reported that constitutive activity of STAT3 up-regulated VEGF expression and tumor angiogenesis [49]. Wei and colleagues also reported that overexpression of constitutively activated mutant STAT3 sufficiently increased VEGF expression and tumor angiogenesis *in vivo* [50]. In contrast, dominant negative STAT3 mutant inhibited VEGF expression as well as angiogenesis. Additionally, it is of interest that STAT3 activation by its upstream Src regulates VEGF mediated angiogenesis and conversely blocking STAT3 inhibits Src-induced VEGF expression [51]. Consistently, in the present study, EP significantly prevented VEGF-induced phosphorylation of STAT3 as well as VEGF-mediated tube formation in HUVECs, indicating anti-angiogenic activity of EP by inhibiting STAT3 phosphorylation. Furthermore, we confirmed anti-tumor effect of EP in mouse xenograft tumor model. Consistent with the results of *in vitro* experiments, EP decreased U266 tumor growth as well as suppressed the expression levels of phospho-STAT3 and CD34 by immunohistochemistry. Although there are evidences that EP induces apoptosis at concentrations of 12.5-50  $\mu$ M in LNCaP and DU-145 cells for 72 h [41,42,52], in the current study, EP did not show any cytotoxicity against U266 cells for 24 h up to 50  $\mu$ M. Actually, immunohistochemistry revealed that TUNEL positive cells were increased in EP treated tumor sections, implying that EP can exert anti-angiogenic activity at non-toxic concentrations and possibly induce apoptosis only after long term culture or at high doses. Thus, mechanism and pharmacokinetic studies with EP are still required *in vitro* and *in vivo* to elucidate the relationship between its anti-angiogenic and apoptotic activities at different doses in the near future

## Conclusions

Our findings demonstrate that EP can exert antitumor activity in multiple myeloma U266 cells by anti-angiogenic activity targeting JAK2/STAT3 signaling pathway as a potent anti-cancer agent for multiple myeloma treatment.

## Acknowledgements

This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (No. 2011-0006220).

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## Authors' contributions

YHR SJJ and SHK SA designed the research studies; YHR, HJeL and HJuL carried out the experiments; YHR SJJ and SHK analyzed and interpreted the data; YHR SJJ and SHK wrote the draft of the manuscript. All authors read and approved the final manuscript.

### Competing interests

The authors declare that they have no competing interests.

Received: 8 July 2011 Accepted: 20 January 2012

Published: 20 January 2012

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#### Pre-publication history

The pre-publication history for this paper can be accessed here:  
<http://www.biomedcentral.com/1471-2407/12/28/prepub>

doi:10.1186/1471-2407-12-28

**Cite this article as:** Rhee *et al*: Inhibition of STAT3 signaling and induction of SHP1 mediate antiangiogenic and antitumor activities of ergosterol peroxide in U266 multiple myeloma cells. *BMC Cancer* 2012 **12**:28.

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