Carcinogenesis vol.28 no.4 pp.858–864, 2007
doi:10.1093/carcin/bgl205
Advance Access publication October 27, 2006

Apigenin inhibits tumor angiogenesis through decreasing HIF-1α and VEGF expression

Jing Fang[1], Qiong Zhou[1], Ling-Zhi Liu[1], Chang Xia[2], Xiaowen Hu[1], Xianglin Shi[1] and Bing-Hua Jiang[1,2,*]

[1]The Institute for Nutritional Sciences, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China and [2]Mary Babb Randolph Cancer Center, Department of Microbiology, Immunology and Cell Biology, West Virginia University, Morgantown, WV 26506, USA

*To whom correspondence should be addressed.
Email: bhjiang@hsc.wvu.edu

Apigenin is a non-toxic dietary flavonoid with anti-tumor properties. We recently showed that apigenin-inhibited hypoxia-inducible factor-1 (HIF-1) and vascular endothelial growth factor (VEGF) expression in human ovarian cancer cells under normoxic condition. However, the effect of apigenin in angiogenesis remains to be elucidated. Angiogenesis is the formation of new blood vessels and is required for tumor growth and metastasis. In this study, we showed that apigenin-inhibited expression of HIF-1 and VEGF in different cancer cells under both normoxic and hypoxic conditions. We demonstrated that apigenin significantly inhibited tumor angiogenesis *in vivo,* by using both the chicken chorioallantoic membrane and Matrigel plug assays. The inhibition of tumor angiogenesis was associated with the decrease of HIF-1 and VEGF in tumor tissues. Taken together, our results show that apigenin suppresses tumor angiogenesis through HIF-1 and VEGF expression.

Introduction

Angiogenesis is the formation of new blood vessels from preexisting ones, and is required for tumor growth and metastasis (1). Tumor angiogenesis is stimulated by angiogenic inducers such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor (TGF) and interleukin 8 (IL-8). VEGF and its receptors are major regulators of angiogenesis (2), and are important in tumor progression (3,4). VEGF expression and its receptor function are required for tumor growth, invasion and metastasis (5–8), and are associated with the development of many human cancers (9). VEGF may regulate both tumor growth and angiogenesis (9–11). Thus, inhibition of VEGF production and/or function is a promising strategy for cancer therapy and prevention.

Hypoxia-inducible factor-1 (HIF-1) activates the transcription of many genes including VEGF (12). HIF-1 activates the expression of *VEGF* gene by binding to the hypoxia response element (HRE) in the VEGF promoter region (13). HIF-1 expression is elevated in many human cancers (14), and HIF-1 levels in cells correlate with tumorigenicity and angiogenesis (15). HIF-1 is composed of HIF-1α and HIF-1β subunits (16,17). In most experimental systems, HIF-1α protein is constitutively expressed, but rapidly degraded by the ubiquitin–proteasome pathway under normoxia condition (18,19), which is mediated by the specific binding of the von Hippel–Lindau (VHL) tumor suppressor to HIF-1α (20). The prolyl hydroxylation of HIF-1α, is required for the HIF-1α–pVHL physical interaction, which is critical in degrading HIF-1α in the cells (21,22). Under hypoxic condition, the absence of oxygen inhibits the hydroxylase activities for modifying HIF-1α, and pVHL can not bind to HIF-1α.

Apigenin (4′,5,7,-trihydroxyflavone) is a common dietary flavonoid which is widely distributed in many fruits and vegetables (23). Apigenin has recently been shown to possess anti-tumor properties against cancers, especially prostate cancer (24–28). We found recently that apigenin is able to inhibit HIF-1 and VEGF expression in ovarian cancer cells under normoxia condition (29). Hypoxia is a major inducer of HIF-1 and VEGF expression. In the present work, we tested whether apigenin (i) inhibits HIF-1 and VEGF expression in other cancer cells; (ii) affects hypoxia-induced HIF-1 and VEGF expression; and (iii) affects tumor angiogenesis *in vivo*.

Abbreviations: β-gal, β-galactosidase; CAM, chicken chorioallantoic membrane; CHX, cycloheximide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Hsp90, heat shock protein 90; HIF-1, hypoxia-inducible factor 1; Luc, luciferase; PI3K, phosphatidylinositol 3-kinase; VEGF, vascular endothelial growth factor.

Materials and methods

Reagents and antibodies

Apigenin and cycloheximide (CHX), an inhibitor of protein synthesis, were purchased from Sigma (St Louis, MO). Apigenin was dissolved in DMSO and stored at −20°C. The antibodies against β-actin and FITC-conjugated second antibodies were also from Sigma. Antibodies against VEGF were obtained from Santa Cruz (Santa Cruz, CA). Antibodies against HIF-1α, HIF-1β, heat shock protein-90 (Hsp90), and the growth-factor-reduced Matrigel were from BD Biosciences (Bedford, MA).

Cell culture

The human prostate cancer cells, PC-3, DU145 and LNCaP; human ovarian cancer cells, OVCAR-3, and human colon cancer cells, HCT-8, were cultured in RPMI 1640 medium. Human breast cancer cells, MCF-7, were cultured in DMEM medium. Both media were supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS; Gibco BRL, Grant Island, NY), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were incubated in a 5% CO_2 incubator at 37°C. For hypoxia treatment, the cells were incubated in a chamber containing 1% oxygen, 5% carbon dioxide and 94% nitrogen at 37°C.

Construction of plasmids

VEGF promoter reporter pMAP11wt containing 47 bp of human VEGF 5′-flanking sequence (from −985 to −939) was inserted into the pGL2 basic luciferase vector as described previously (13). The mutant VEGF promoter reporter pMAP11mut was constructed by introducing a 3-bp substitution at the HIF-1 binding site (13). The plasmid encoding human HIF-1α was inserted into pCEP4 vector as we described previously (13,16).

Immunoblotting, immunoprecipitation, and ELISA analysis

Immunoblotting analysis of HIF-1α and other proteins was performed as described previously (30). For immunoprecipitation, cellular proteins (500 μg) were incubated with 1 μg of anti-Hsp90 antibodies at 4°C for 3 h, then with 30 μl of protein A/G plus-agarose at 4°C overnight. The beads were washed four times with ice-cold phosphate-buffered saline (PBS), then incubated with 30 μl of SDS–PAGE loading buffer and heated at 100°C for 3 min. The protein samples were resolved on 8% SDS–PAGE for immunoblotting. VEGF protein levels were determined using the Quantikine human VEGF ELISA kit from R&D Systems (Minneapolis, MN) as described previously (30). In brief, the cells were seeded in 12-well plates, and cultured to 90–100% confluence. The cells were switched to fresh medium in the presence or absence of apigenin, and incubated under hypoxic or normoxic conditions for the time period as indicated. After the treatment, the supernatants were collected and the cells in each well were counted. Relative VEGF concentrations in the supernatant (100 μl) were determined and normalized to the cell number in each well with a serial dilution of human recombinant VEGF as a standard curve.

Transient transfection and luciferase assay

The cells were seeded in 6-well plates, and cultured to 60–70% confluence. To determine the effect of apigenin on VEGF transcriptional activation, the cells were transiently transfected with VEGF reporter and pCMV-β-galactosidase plasmid using Lipofectamine (Invitrogen, Carlsbad, CA). The transfected cells were cultured for 20 h, followed by incubation with apigenin for 15 h. Cells were then washed once with PBS, and lysed with reporter lysis buffer from Promega (Madison, WI). The luciferase (Luc) activities were determined as described by the authors previously (30). The relative Luc activity was calculated as the ratio of Luc/β-gal activity, and normalized to that of the control.

Tumor angiogenesis assay using chicken chorioallantoic membrane (CAM)

To investigate the effect of apigenin on tumor-induced angiogenesis, a modified CAM assay was carried out. Briefly, the cells were trypsinized, washed and re-suspended in serum-free RPMI 1640 medium at 4°C. The cells were mixed with 50% (v/v) of growth-factor-reduced Matrigel in the absence or presence of apigenin. The mixture (30 μl) was loaded onto the CAM of a 9-day-old chicken embryo. After the incubation for 4 days, the area around the implantation was photographed with a Nikon digital camera, and the blood vessels were determined by two observers in a double-blind manner.

Matrigel plug assay

Male and female BALB/cA-nu nude mice (4-weeks-old) were purchased from Shanghai Experimental Animal Center (Chinese Academy of Sciences, Shanghai, China), and maintained in pathogen-free conditions. The cells at subconfluence were harvested, washed with PBS and re-suspended in serum-free medium. Aliquots of the cells (0.2 ml) were mixed with 0.4 ml of Matrigel in the presence or absence of apigenin. The mixture was immediately injected into both flanks of nude mice. Male and female nude mice were injected with PC-3 and OVCAR3 cells, respectively. The mice were sacrificed when tumors were visible, and the Matrigel plugs were carefully taken out of the mice without other adjacent tissues. The excised plugs were placed in cold PBS at 4°C overnight to be liquefied. Specimens were centrifuged at 14 000 r.p.m. to collect the supernatant. Hemoglobin contents were quantified using Drabkin's reagent kit (Sigma-Aldrich, St Louis, MO) as described previously (31).

Immunohistochemical staining for VEGF

Tumor tissues were fixed in 4% buffered formalin for 24 h, and processed by conventional paraffin-embedded method. The paraffin-embedded tumor sections (5-μm thick) were heat-immobilized, deparaffinized using xylene, then rehydrated in a series of increasing ethanol concentrations. Antigen retrieval was done by incubating the sections in 10 mM citrate buffer (pH 6.0) in microwave for 10 min, followed by the incubation with 1.5% block serum for 1 h. Sections were then incubated with antibodies against VEGF at 4°C overnight, followed by the incubation with the FITC-conjugated second antibodies for 1 h at room temperature in the dark. The tissue sections were stained with propidium iodide for a short time, and subsequently detected under a confocal microscope.

Statistical analysis

The data represent mean ± SD from three independent experiments except those that are specifically indicated. Statistical analysis was performed using Student *t* test.

Results

Apigenin inhibited HIF-1α and VEGF expression in PC-3 and DU145 cells under normoxia and hypoxia conditions

It has been reported that overexpression of HIF-1α is commonly observed in many human cancers including prostate, breast, colon, and ovarian cancer cells (32,33). To test whether apigenin could suppress HIF-1α expression in human prostate cancer cells, PC-3 and DU145 cells were treated with apigenin. As shown in Figure 1A, PC-3 and DU145 cells produced high levels of HIF-1α protein under normoxic condition (21% O_2), and apigenin significantly inhibited HIF-1α expression. Apigenin had little effect on HIF-1β expression. Similarly, PC-3 and DU145 cells produced high levels of VEGF protein and apigenin suppressed VEGF expression (Figure 1B). To further assess the effect of apigenin under hypoxic condition (1% oxygen), HIF-1α and VEGF protein levels were determined in PC-3 cells. Hypoxia increased the production of VEGF protein and apigenin treatment inhibited the hypoxia-induced VEGF production (Figure 1C). Immunoblotting analysis showed that hypoxia-induced HIF-1α expression, which was inhibited by apigenin treatment (Figure 1C). These data indicated that apigenin exerts its inhibitory effect on HIF-1α and VEGF expression under both normoxic and hypoxic conditions.

To know whether apigenin inhibited VEGF transcriptional activation, PC-3 and DU145 cells were transfected with VEGF promoter reporter and treated with apigenin. Apigenin inhibited VEGF reporter activity in both PC-3 and DU145 cells (Figure 1D). Apigenin did not affect cell viability under the same experimental conditions (data not shown) as described previously (29). To test whether apigenin inhibits VEGF through HIF-1α expression, the cells were co-transfected with VEGF reporter and HIF-1α plasmids. Overexpression of HIF-1α reversed apigenin inhibited VEGF reporter activities in both PC-3 and DU145 cells (Figure 1E). In order to determine whether apigenin suppresses VEGF transcriptional activation through HIF-1α binding site at the VEGF promoter, a mutant VEGF promoter reporter, pMAP11mut was used to test the effect of apigenin (13). Treatment of the cells with apigenin did not inhibit the mutant reporter activity (data not shown), indicating that apigenin affects VEGF transcriptional activation through the HIF-1 binding site. These results suggest that apigenin inhibits VEGF transcriptional activation through HIF-1α-ha*expression*.

Apigenin inhibited hypoxia-induced HIF-1α and VEGF expression in different cancer cells

To test whether apigenin affects hypoxia-induced HIF-1α and VEGF expression, we use cancer cell lines with low level of HIF-1α expression under normoxia condition. Human prostate cancer, LNCaP cells, human colon cancer, HCT-8 cells, and human breast cancer, MCF-7 cells, produced low levels of HIF-1α and VEGF proteins in normal cultured condition (Figure 2). However, when cells were incubated under hypoxic condition, levels of HIF-1α protein were significantly induced in the cells (Figure 2A). Treatment of the cells with apigenin abrogated the hypoxia induction of HIF-1α protein (Figure 2A). Similarly, hypoxia dramatically induced VEGF production and apigenin inhibited hypoxia-induced expression of VEGF (Figure 2B). Since hypoxia is associated with tumor growth, the inhibitory effect of

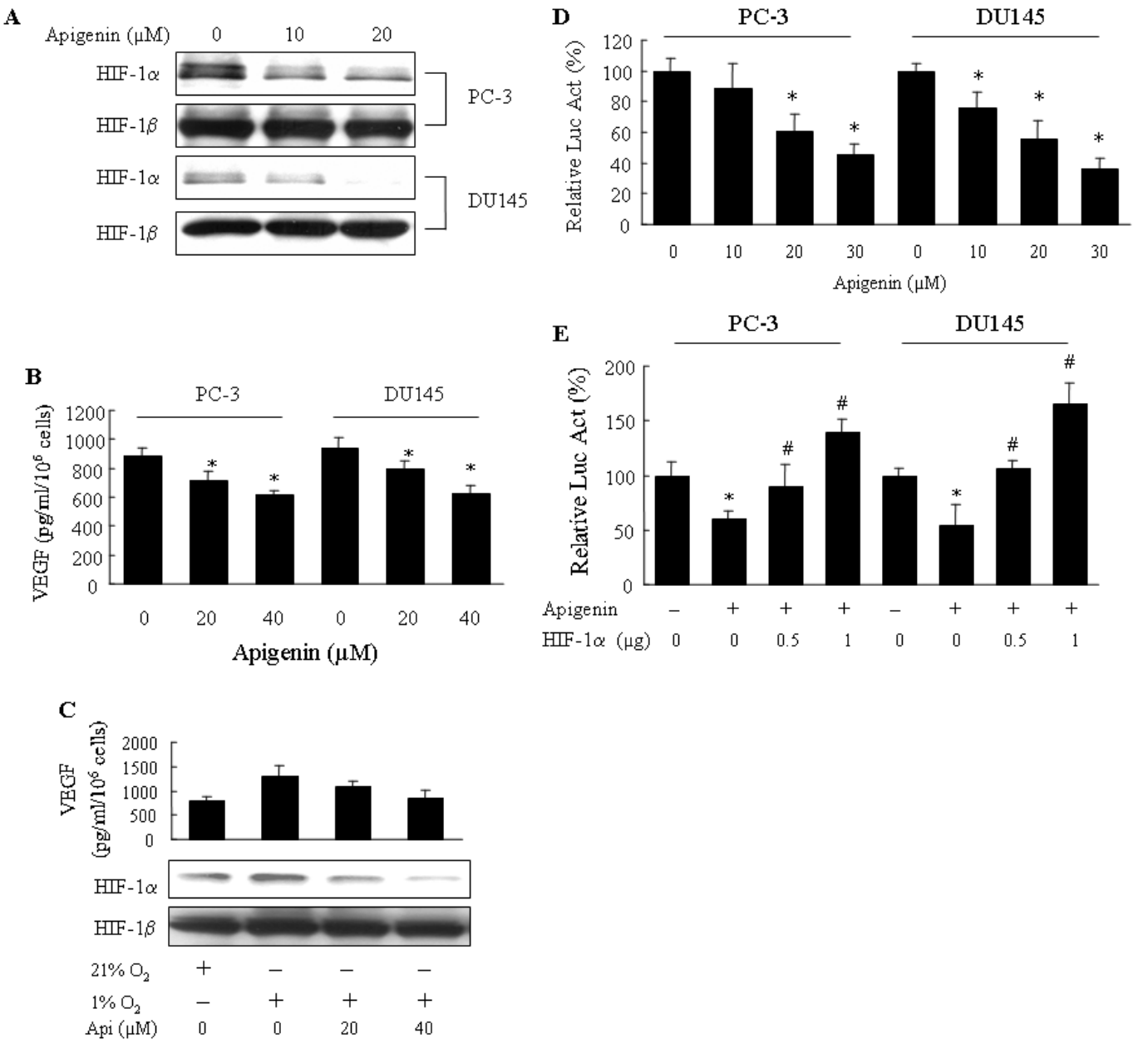

Fig. 1. Apigenin inhibited HIF-1α and VEGF expression in PC-3 and DU145 cells. (**A**) PC-3 and DU145 cells were cultured to 80–90% confluence, followed by a 6-h treatment with apigenin as indicated. The cells treated with solvent alone were used as a control. HIF-1α and HIF-1β protein levels were detected by immunoblotting as described in Materials and methods. (**B**) Apigenin suppresses VEGF production in PC-3 and DU145 cells. The cells were seeded in 12-well plates, and cultured to 90–100% confluence. The old medium was discarded and fresh medium with or without apigenin was added. The cells were incubated for 15 h. The VEGF concentrations in the medium were measure by ELISA. *, Indicates significant decrease of VEGF when compared to that of control ($P < 0.05$). (**C**) Apigenin (Api)-inhibited HIF-1α and VEGF expression under hypoxic condition. For determination of VEGF production, the cells were seeded in 12-well plates, and cultured to 90–100% confluence. The old medium was discarded and fresh medium with or without apigenin was added. The cells were incubated under hypoxia for 18 h. VEGF was measured as described previously. For immunoblotting analysis, PC-3 cells were pretreated with apigenin for 1 h, followed by the incubation for 6 h under hypoxic condition. The cells were harvested and lysed. Total proteins were analyzed by immunoblotting. *, Indicates significant decrease of VEGF levels when compared to that of the control ($P < 0.05$). (**D**) Apigenin inhibited VEGF transcriptional activation. The cells were transfected with 1 μg of human VEGF promoter reporter, pMAP11wt and 0.3 μg of β-gal plasmid, and cultured for 20 h, then treated with apigenin for 15 h. Cells were lysed and the supernatants were used for Luc and β-gal activity assay. The relative Luc activities in the cell extracts were assayed by the ratio of Luc/β-gal activity, and normalized to the value of the solvent DMSO control. *, Indicates significant difference when the value is compared to the control ($P < 0.05$). (**E**) Overexpression of HIF-1α reversed inhibitory effects of apigenin on VEGF transcriptional activation. PC-3 and DU145 cells were transfected with 1 μg of pMAP11wt reporter, 0.3 μg of β-gal plasmid and 0.5 μg (or 1 μg) of wild-type HIF-1α plasmid. The empty vector was added to make total transfected DNA to 2.3 μg. The concentration of apigenin used was 20 μM. *, Indicates significant difference when the value is compared to the control, and, hash symbol, indicates significant difference when the value is compared to the apigenin treatment alone ($P < 0.05$).

apigenin on HIF-1α and VEGF expression is an important factor for inhibiting tumor growth.

To study the potential mechanism that apigenin inhibits HIF-1α expression, the cells were treated by apigenin in the presence of cycloheximide for inhibiting *de novo* protein synthesis. Apigenin treatment reduced the stability of HIF-1α in PC-3 cells (Figure 3A). The binding of Hsp90 and HIF-1α is known to stabilize HIF-1α protein (34–36). We therefore

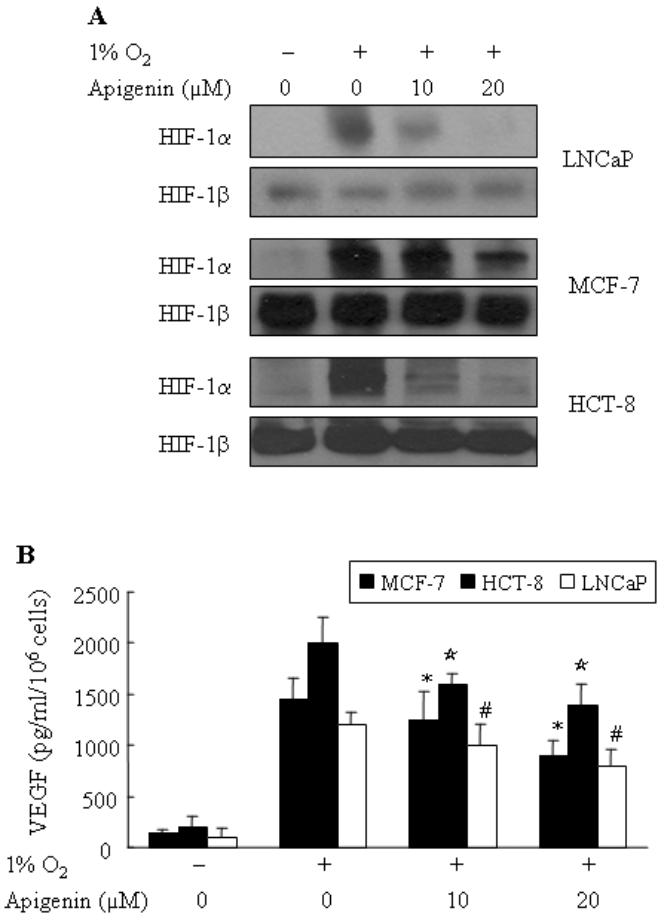

Fig. 2. Apigenin inhibited HIF-1α and VEGF expression in HCT-8, MCF-7, and LNCaP cells under hypoxia. (**A**) LNCaP, HCT-8 and MCF-7 cells at 80–90% confluence were pretreated with apigenin for 1 h, then incubated under hypoxic condition for 6 h. HIF-1expression was determined by immnoblotting. (**B**) The cells were seeded in 12-well plates and cultured to 90–100% confluence. The old medium was discarded and fresh medium with or without apigenin was added. The cells were incubated for 18 h under hypoxia. VEGF in the medium was determined by ELISA. Asterisks, hash symbols and open squares indicate significant decrease of VEGF level when compared to the solvent control of each cell line ($P < 0.05$).

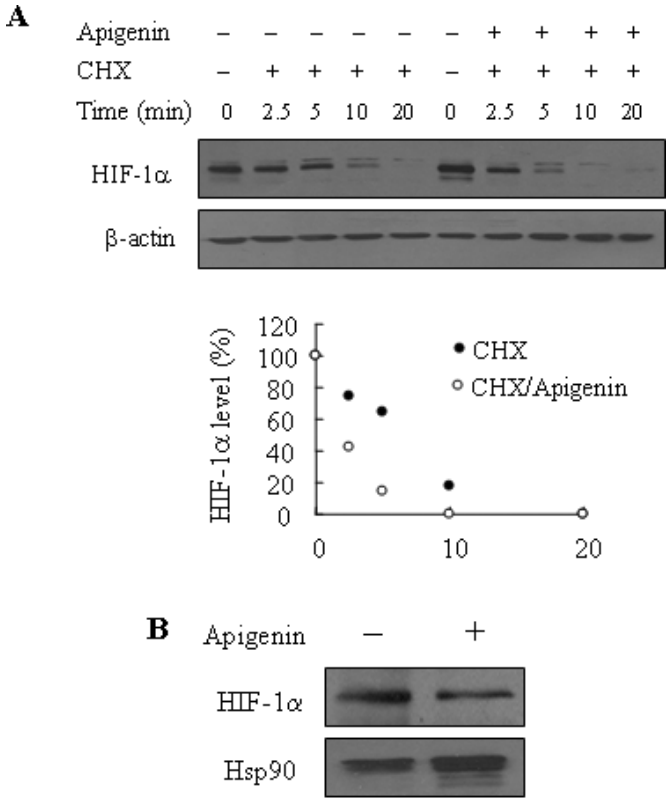

Fig. 3. Apigenin reduced stability of HIF-1α and interfered the binding of HIF-1α and Hsp90. (**A**) PC-3 cells were cultured to 80–90% confluence in normal medium. The cells were pretreated without or with apigenin (20 μM) for 1 h, then incubated with CHX (100 μM) in the presence or absence of apigenin for different times as indicated. HIF-1α and β-actin levels were detected by immunoblotting. Relative levels of HIF-1α protein were determined by measuring the density of the HIF-1α protein band and normalized to that of β-actin. The relative HIF-1α protein level in the control is defined as 100%. (**B**) PC-3 cells cultured in normal medium were harvested when the cells were at 80–90% confluence. The cell lysates (500 μg) were incubated with 1 μg Hsp90 antibodies in the presence of solvent alone or apigenin (40 μM) for 3 h at 4°C. Aliquots of the Protein A/G plus-agarose beads were added to the lysates, and incubated overnight. The beads were washed four times with ice-cold PBS, then incubated with SDS–PAGE loading buffer, and heated at 100°C for 3 min. The proteins were resolved on 8% SDS–PAGE, and analyzed by immunoblotting.

determined the effects of apigenin on the interaction between Hsp90 and HIF-1α. As shown in Figure 3B, apigenin inhibited the binding of HIF-1α and Hsp90. These results suggest that apigenin decreases HIF-1α level by interfering the binding of HIF-1α and Hsp90 in the cancer cells.

Apigenin inhibited tumor angiogenesis in vivo

To assess whether apigenin inhibits angiogenesis *in vivo*, we used CAM model to study the effect of apigenin on tumor-induced angiogenesis. As indicated in Figure 4A, PC-3 and OVCAR-3 cells induced angiogenesis on the CAM. Treatment of the cells with apigenin inhibited the tumor-induced angiogenesis (Figure 4A). To quantify the relative angiogenesis on the CAM, the blood vessels were counted in replicate experiments. As shown in Figure 4B, PC-3 and OVCAR-3 cells increased the relative angiogenesis 3- and 4-fold, respectively. The tumor-induced angiogenesis was significantly suppressed by apigenin treatment in a dose-dependent manner (Figure 4B). We analyzed VEGF expression in the tumor tissues by immunohistochemistry staining. Apigenin treatment greatly decreased the VEGF-positive signals in the tumor tissues (Figure 4C). We also analyzed HIF-1α protein levels in tumor tissues using the extracts of the tumor tissues by immunoblotting. Apigenin treatment inhibited HIF-1α expression in the tumors (Figure 4D). These results suggest that apigeinin suppress tumor angiogenesis by inhibiting expression of HIF-1α and VEGF in the tumors. To determine the signaling molecules that might be involved, we determined the phosphorylation of AKT and p70S6K1 in the extracts prepared from tumor tissues, and found that the phosphorylation levels of AKT and p70S6K1 were suppressed by apigenin treatment (Figure 4D).

To further analyze the effect of apigenin in angiogenesis in a different animal model, we studied angiogenesis by Matrigel plug assay in nude mice. Similarly, we used PC-3 and OVCAR-3 cells for the study, and found that PC-3 and OVCAR-3 cells greatly induced the formation of blood vessels in the Matrigel plug (Figure 5A). Apigenin treatment inhibited the tumor cell-induced angiogenesis (Figure 5A). Hemoglobin contents in the plug were determined as a relative angiogenesis index. There was little hemoglobin in

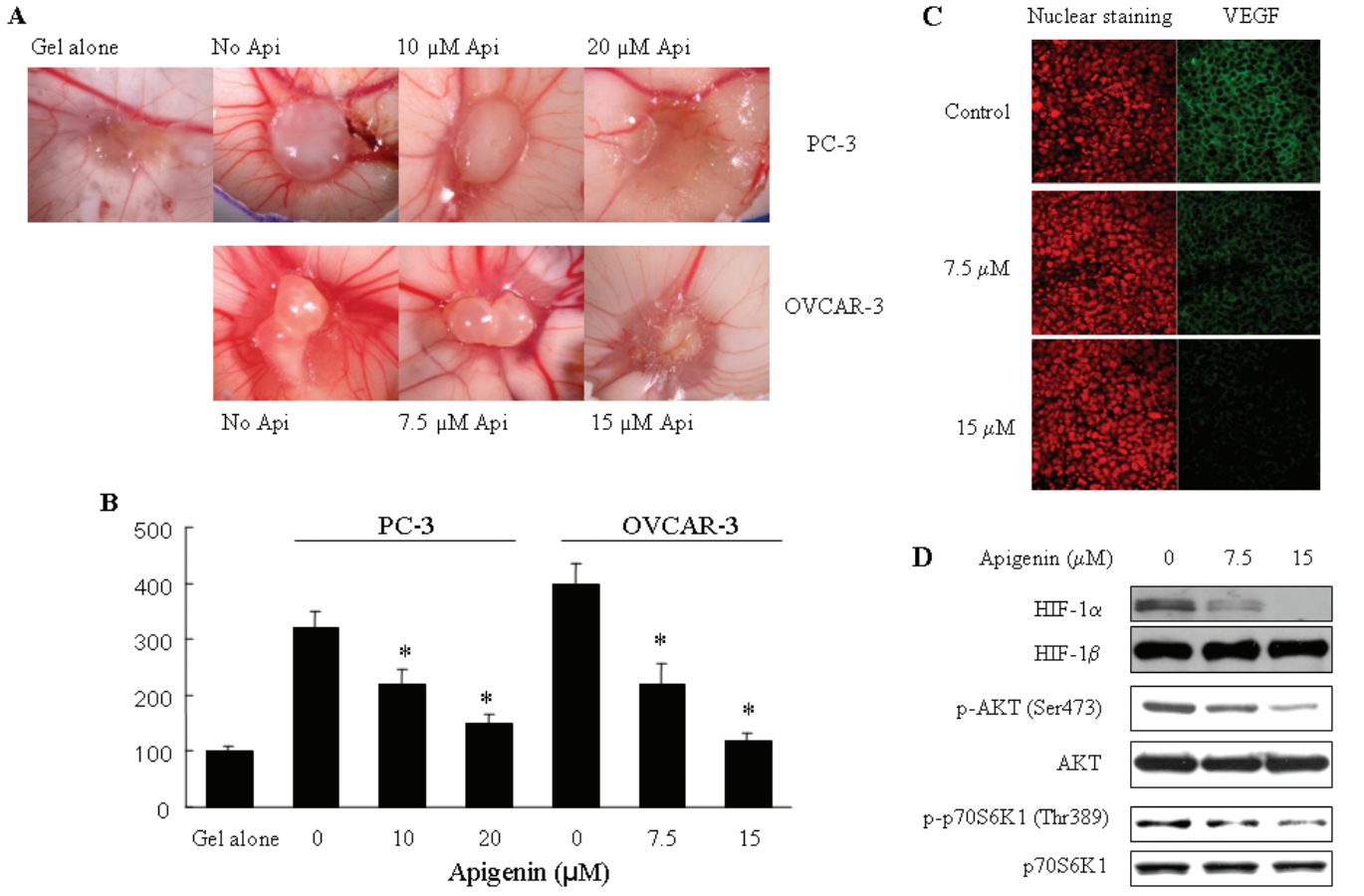

Fig. 4. Apigenin inhibited *in vivo* tumor angiogenesis. (**A**) Apigenin inhibited tumor angiogenesis in the CAM model. PC-3 and OVCAR-3 cells were harvested, and re-suspended in serum-free medium at 1.5×10^7 cells/ml. Aliquots of the cells (15 μl) were mixed with 15 μl of growth-factor-reduced Matrigel in the absence or presence of apigenin as indicated. The cell mixture was implanted onto the CAM of a 9-day-old chick embryo to grow tumor. The total blood vessels around the Matrigel were counted double-blindly under a microscope 4 days after the implantation. The pictures are representatives of each treatment. (**B**) Quantification of blood vessels on the CAM. The number of blood vessels was normalized to that of Matrigel alone, and the data was the mean ± SD from two independent experiments ($n = 8$). *, Indicated significant difference when the blood vessel number was compared to that of the cells without apigenin ($P < 0.05$). (**C**) Immunohistochemical staining of VEGF expression in the CAM tumor tissues induced by OVCAR-3 cells. (**D**) Phosphorylation of AKT and p70S6K1 in the tissue extracts. The OVCAR-3-induced tumors on the CAM were snap-frozen in liquid nitrogen, and total tissue extracts were prepared in RIPA lysis buffer and analyzed by immunoblotting.

the Matrigel plug alone. The hemoglobin contents in the plugs with PC-3 and OVCAR-3 cells were 7 and 12 mg/g, respectively (Figure 5B). Apigenin treatment inhibited the hemoglobin levels by >50% in both PC-3- and OVCAR-3-induced tumors (Figure 5B). Taken together, these results indicated that apigenin is a potent inhibitor of tumor angiogenesis.

Discussion

Angiogenesis is required for tumor growth and metastasis. Without new blood vessel formation, the tumor cannot grow larger than 1–2 mm in diameter. Therefore, the development of anti-angiogenesis reagents has become a new attractive strategy to combat cancer. Angiogenesis is stimulated by a few growth factors among which VEGF plays a fundamental role. In human cancers, VEGF expression has been related to a more aggressive (37,38), and metastatic phenotype (39). Similar results were observed using animal models (40). Inhibition of VEGF production is a promising therapeutic approach for cancer. We recently found that apigenin inhibited expression of HIF-1α and VEGF in ovarian cancer cells *in vitro* (29). However, it is not known that apigenin also inhibits HIF-1α and VEGF production in other cancer cells and it is not clear whether apigenin inhibits *in vivo* tumor angiogenesis. In this study, we tested several human cancer cells including prostate cancer cells PC-3, DU145 and LNCaP, breast cancer cell MCF-7, and colon cancer cell HCT-8. PC-3 and DU145 cells produced high levels of HIF-1α and VEGF under normoxic conditions (Figure 1A and B). Apigenin inhibited expression of HIF-1α and VEGF in these cells (Figure 1A and B). We found that apigenin inhibited VEGF transcriptional activation through HIF-1α expression (Figure 1D and E). During tumor growth, there is low oxygen environment inside the tumor. Therefore, it is interesting to know whether apigenin could inhibit HIF-1α and VEGF expression induced by hypoxia. Under hypoxia condition, accumulation of HIF-1α and VEGF proteins was observed in PC-3 cells and addition of apigenin suppressed hypoxia-induced HIF-1α and VEGF expression (Figure 1C). Similarly, the induction of HIF-1α and VEGF by hypoxia was abrogated in several different cancer cells by apigenin treatment (Figure 2). These results suggest that apigenin inhibits HIF-1α and VEGF expression under both normoxic and hypoxic conditions in different cancer cells.

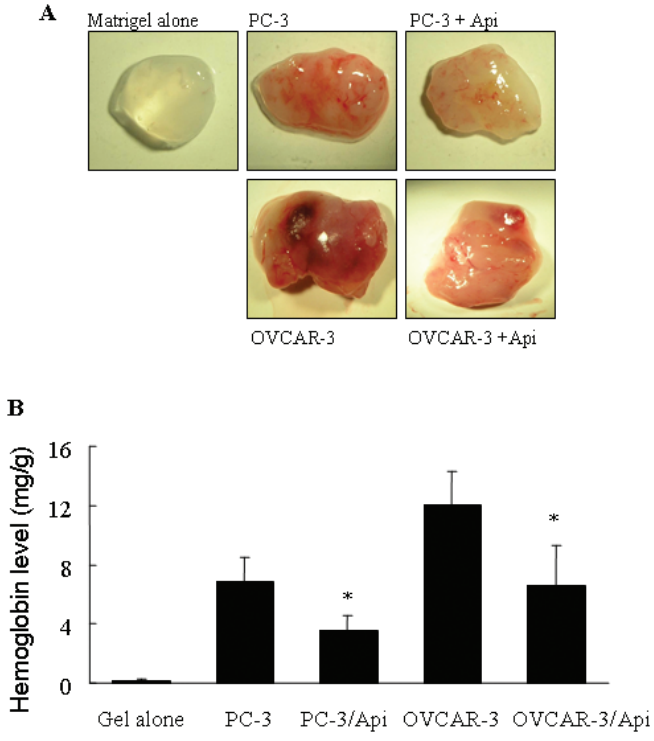

Fig. 5. Apigenin inhibited tumor angiogenesis in nude mice model. PC-3 and OVCAR-3 cells were re-suspended in serum-free medium at 1.5×10^7 cells/ml, and aliquots of the cells (0.2 ml) were mixed with 0.4 ml of growth-factor-reduced Matrigel in the absence or presence of apigenin (Api) at 20 μM (PC-3 cells) or at 15 μM (OVCAR-3 cells). The PC-3 and OVCAR-3 cells with Matrigel were injected subcutaneously into the both sites of male and female mice, respectively. The male and female nude mice were euthanized in 11 days and 7 days, respectively, after the implantation. (**A**) The Matrigel plugs were removed from the mice. The photos are representative Matrigel plugs. (**B**) Hemoglobin levels of Matrigel plugs. Hemoglobin levels in the supernatant were quantified with Drabkin's reagent kit (Sigma-Aldrich, St Louis, MO). The data are mean ± SD from replicate experiments ($n = 10$). *, Indicated significant difference when the hemoglobin content was compared to that of the control ($P < 0.05$).

To assess the effects of apigenin on tumor angiogenesis, we performed angiogenesis assay *in vivo* using two different assay systems. The results showed that apigenin is a potent inhibitor of tumor-induced angiogenesis (Figures 4 and 5). Apigenin also inhibited expression of HIF-1α and VEGF in tumor tissues (Figure 4C and D), suggesting that apigenin inhibits angiogenesis through HIF-1α and VEGF expression. Expression of HIF-1α may be regulated through protein synthesis and degradation pathways (33). Under normoxia condition, HIF-1α expression is induced via activation of PI3K/AKT and MAPK signaling, which is induced by growth factors, cytokines and other signaling molecules (41–44), and/or oncogenic mutations of some tumor suppressor genes, such as *VHL* (20,45), *PTEN* (45) and *p53* (46). Under hypoxia condition, the accumulation of HIF-α is due to the inhibition of HIF-1α degradation (33). We found that apigenin reduced HIF-1α stability by blocking the binding of HIF-1α and Hsp90 (Figure 3). It is known that Hsp90 binding stabilizes HIF-1α protein (34,36). Out results suggest that apigenin inhibits HIF-1α expression through interfering the interaction between HIF-1α and Hsp90. The analysis of tumor tissues indicated that apigenin inhibited phosphorylation of AKT (Figure 4D), suggesting that apigenin may also inhibit HIF-1α expression via AKT signaling. These *in vivo* data are consistent with our previous *in vivo* results (29).

Within the solid tumor, hypoxia commonly develops because the rate of tumor cell proliferation outpaces the rate of blood vessel formation. This study shows that apigenin can inhibit expression of HIF-1α and VEGF in cancer cells under both hypoxia and normoxic conditions, suggesting that apigenin is a potent inhibitor of tumor angiogenesis. The plant-based diets on prevention of cancer and other chronic diseases have been well documented (24). In the present work, we demonstrate that apigenin inhibited VEGF expression through HIF-1 expression in various cancer cells, and suppressed angiogenesis *in vivo*. These results suggest that apigenin may be used as a chemopreventive and/or chemotherapeutic agent against human cancer in the future.

Conflict of Interest Statement: None declared.

References

1. Folkman,J. (2002) Role of angiogenesis in tumor growth and metastasis. *Semin. Oncol.*, **29**, 15–18.
2. Plate,K.H., Breier,G., Weich,H.A. and Risau,W. (1992) Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas *in vivo*. *Nature*, **359**, 845–848.
3. Ferrara,N. and Davis-Smyth,T. (1997) The biology of vascular endothelial growth factor. *Endocr. Rev.*, **18**, 4–25.
4. Carmeliet,P. and Jain,R.K. (2000) Angiogenesis in cancer and other diseases. *Nature*, **407**, 249–257.
5. Brekken,R.A., Overholser,J.P., Stastny,V.A., Waltenberger,J., Minna,J.D. and Thorpe,P.E. (2000) Selective inhibition of vascular endothelial growth factor (VEGF) receptor 2 (KDR/Flk-1) activity by a monoclonal anti-VEGF antibody blocks tumor growth in mice. *Cancer Res.*, **60**, 5117–5124.
6. Borgstrom,P., Bourdon,M.A., Hillan,K.J., Sriramarao,P. and Ferrara,N. (1998) Neutralizing anti-vascular endothelial growth factor antibody completely inhibits angiogenesis and growth of human prostate carcinoma micro tumors *in vivo*. *Prostate*, **35**, 1–10.
7. Angelov,L., Salhia,B., Roncari,L., McMahon,G. and Guha,A. (1999) Inhibition of angiogenesis by blocking activation of the vascular endothelial growth factor receptor 2 leads to decreased growth of neurogenic sarcomas. *Cancer Res.*, **59**, 5536–5541.
8. Cheng,S.Y., Huang,H.J., Nagane,M., Ji,X.D., Wang,D., Shih,C.C., Arap,W., Huang,C.M. and Cavenee,W.K. (1996) Suppression of glioblastoma angiogenicity and tumorigenicity by inhibition of endogenous expression of vascular endothelial growth factor. *Proc. Natl Acad. Sci. USA*, **93**, 8502–8507.
9. Masood,R., Cai,J., Zheng,T., Smith,D.L., Hinton,D.R. and Gill,P.S. (2001) Vascular endothelial growth factor (VEGF) is an autocrine growth factor for VEGF receptor-positive human tumors. *Blood*, **98**, 1904–1913.
10. Jackson,M.W., Roberts,J.S., Heckford,S.E., Ricciardelli,C., Stahl,J., Choong,C., Horsfall,D.J. and Tilley,W.D. (2002) A potential autocrine role for vascular endothelial growth factor in prostate cancer. *Cancer Res.*, **62**, 854–859.
11. Soker,S., Kaefer,M., Johnson,M., Klagsbrun,M., Atala,A. and Freeman,M.R. (2001) Vascular endothelial growth factor-mediated autocrine stimulation of prostate tumor cells coincides with progression to a malignant phenotype. *Am. J. Pathol.*, **159**, 651–659.
12. Semenza,G.L. (2000) Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. *Crit. Rev. Biochem. Mol. Biol.*, **35**, 71–103.
13. Forsythe,J.A., Jiang,B.H., Iyer,N.V., Agani,F., Leung,S.W., Koos,R.D. and Semenza,G.L. (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol. Cell. Biol.*, **16**, 4604–4613.
14. Zhong,H., De Marzo,A.M., Laughner,E., Lim,M., Hilton,D.A., Zagzag,D., Buechler,P., Isaacs,W.B., Semenza,G.L. and Simons,J.W. (1999) Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res.*, **59**, 5830–5835.
15. Maxwell,P.H., Dachs,G.U., Gleadle,J.M., Nicholls,L.G., Harris,A.L., Stratford,I.J., Hankinson,O., Pugh,C.W. and Ratcliffe,P.J. (1997) Hypoxia-inducible factor-1 modulates gene expression in solid tumors

and influences both angiogenesis and tumor growth. *Proc. Natl Acad. Sci. USA*, **94**, 8104–8109.
16. Jiang,B.H., Rue,E., Wang,G.L., Roe,R. and Semenza,G.L. (1996) Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. *J. Biol. Chem.*, **271**, 17771–17778.
17. Wang,G.L., Jiang,B.H., Rue,E.A. and Semenza,G.L. (1995) Hypoxia-inducible factor 1 is a basic-helix–loop–helix-PAS heterodimer regulated by cellular O2 tension. *Proc. Natl Acad. Sci. USA*, **92**, 5510–5514.
18. Kallio,P.J., Wilson,W.J., O'Brien,S., Makino,Y. and Poellinger,L. (1999) Regulation of the hypoxia-inducible transcription factor 1alpha by the ubiquitin-proteasome pathway. *J. Biol. Chem.*, **274**, 6519–6525.
19. Salceda,S. and Caro,J. (1997) Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J. Biol. Chem.*, **272**, 22642–22647.
20. Maxwell,P.H., Wiesener,M.S., Chang,G.W., Clifford,S.C., Vaux,E.C., Cockman,M.E., Wykoff,C.C., Pugh,C.W., Maher,E.R. and Ratcliffe,P.J. (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature*, **399**, 271–275.
21. Jaakkola,P., Mole,D.R., Tian,Y.M. *et al.* (2001) Targeting of HIF-alpha to the von Hippel–Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science*, **292**, 468–472.
22. Ivan,M., Kondo,K., Yang,H., Kim,W., Valiando,J., Ohh,M., Salic,A., Asara,J.M., Lane,W.S. and Kaelin,W.G. Jr (2001) HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science*, **292**, 464–468.
23. Duthie,G. and Crozier,A. (2000) Plant-derived phenolic antioxidants. *Curr. Opin. Clin. Nutr. Metab. Care*, **3**, 447–451.
24. Fotsis,T., Pepper,M.S., Aktas,E., Breit,S., Rasku,S., Adlercreutz,H., Wahala,K., Montesano,R. and Schweigerer,L. (1997) Flavonoids, dietary-derived inhibitors of cell proliferation and *in vitro* angiogenesis. *Cancer Res.*, **57**, 2916–2921.
25. Morrissey,C., O'Neill,A., Spengler,B., Christoffel,V., Fitzpatrick,J.M. and Watson,R.W. (2005) Apigenin drives the production of reactive oxygen species and initiates a mitochondrial mediated cell death pathway in prostate epithelial cells. *Prostate*, **63**, 131–142.
26. Yin,F., Giuliano,A.E., Law,R.E. and Van Herle,A.J. (2001) Apigenin inhibits growth and induces G_2/M arrest by modulating cyclin-CDK regulators and ERK MAP kinase activation in breast carcinoma cells. *Anticancer Res.*, **21**, 413–420.
27. Gupta,S., Afaq,F. and Mukhtar,H. (2001) Selective growth-inhibitory, cell-cycle deregulatory and apoptotic response of apigenin in normal versus human prostate carcinoma cells. *Biochem. Biophys. Res. Commun.*, **287**, 914–920.
28. Wang,W., Heideman,L., Chung,C.S., Pelling,J.C., Koehler,K.J. and Birt,D.F. (2000) Cell-cycle arrest at G_2/M and growth inhibition by apigenin in human colon carcinoma cell lines. *Mol. Carcinog.*, **28**, 102–110.
29. Fang,J., Xia,C., Cao,Z., Zheng,J.Z., Reed,E. and Jiang,B.H. (2005) Apigenin inhibits VEGF and HIF-1 expression via PI3K/AKT/p70S6K1 and HDM2/p53 pathways. *FASEB J.*, **19**, 342–353.
30. Fang,J., Cao,Z., Chen,Y.C., Reed,E. and Jiang,B.H. (2004) 9-beta-D-arabinofuranosyl-2-fluoroadenine inhibits expression of vascular endothelial growth factor through hypoxia-inducible factor-1 in human ovarian cancer cells. *Mol. Pharmacol.*, **66**, 178–186.
31. Passaniti,A., Taylor,R.M., Pili,R., Guo,Y., Long,P.V., Haney,J.A., Pauly,R.R., Grant,D.S. and Martin,G.R. (1992) A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. *Lab Invest*, **67**, 519–528.
32. Zhong,H., De Marzo,A.M., Laughner,E., Lim,M., Hilton,D.A., Zagzag,D., Buechler,P., Isaacs,W.B., Semenza,G.L. and Simons,J.W. (1999) Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res.*, **59**, 5830–5835.
33. Semenza,G.L. (2003) Targeting HIF-1 for cancer therapy. *Nat. Rev. Cancer*, **3**, 721–732.
34. Katschinski,D.M., Le,L., Heinrich,D., Wagner,K.F., Hofer,T., Schindler,S.G. and Wenger,R.H. (2002) Heat induction of the unphosphorylated form of hypoxia-inducible factor-1alpha is dependent on heat shock protein-90 activity. *J. Biol. Chem.*, **277**, 9262–9267.
35. Minet,E., Mottet,D., Michel,G., Roland,I., Raes,M., Remacle,J. and Michiels,C. (1999) Hypoxia-induced activation of HIF-1: role of HIF-1alpha–Hsp90 interaction. *FEBS Lett.*, **460**, 251–256.
36. Isaacs,J.S., Jung,Y.J., Mimnaugh,E.G., Martinez,A., Cuttitta,F. and Neckers,L.M. (2002) Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1 alpha-degradative pathway. *J. Biol. Chem.*, **277**, 29936–29944.
37. Balbay,M.D., Pettaway,C.A., Kuniyasu,H., Inoue,K., Ramirez,E., Li,E., Fidler,I.J. and Dinney,C.P. (1999) Highly metastatic human prostate cancer growing within the prostate of athymic mice overexpresses vascular endothelial growth factor. *Clin. Cancer Res.*, **5**, 783–789.
38. Connolly,J.M. and Rose,D.P. (1998) Angiogenesis in two human prostate cancer cell lines with differing metastatic potential when growing as solid tumors in nude mice. *J. Urol.*, **160**, 932–936.
39. Melnyk,O., Zimmerman,M., Kim,K.J. and Shuman,M. (1999) Neutralizing anti-vascular endothelial growth factor antibody inhibits further growth of established prostate cancer and metastases in a pre-clinical model. *J. Urol.*, **161**, 960–963.
40. Harper,M.E., Glynne-Jones,E., Goddard,L., Mathews,P. and Nicholson,R.I. (1998) Expression of androgen receptor and growth factors in premalignant lesions of the prostate. *J. Pathol.*, **186**, 169–177.
41. Zhong,H., Chiles,K., Feldser,D., Laughner,E., Hanrahan,C., Georgescu,M.M., Simons,J.W. and Semenza,G.L. (2000) Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res.*, **60**, 1541–1545.
42. Jiang,B.H., Jiang,G., Zheng,J.Z., Lu,Z., Hunter,T. and Vogt,P.K. (2001) Phosphatidylinositol 3-kinase signaling controls levels of hypoxia-inducible factor 1. *Cell Growth Differ.*, **12**, 363–369.
43. Laughner,E., Taghavi,P., Chiles,K., Mahon,P.C. and Semenza,G.L. (2001) HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Mol. Cell. Biol.*, **21**, 3995–4004.
44. Stiehl,D.P., Jelkmann,W., Wenger,R.H. and Hellwig-Burgel,T. (2002) Normoxic induction of the hypoxia-inducible factor 1alpha by insulin and interleukin-1beta involves the phosphatidylinositol 3-kinase pathway. *FEBS Lett.*, **512**, 157–162.
45. Zundel,W., Schindler,C., Haas-Kogan,D. *et al.* (2000) Loss of PTEN facilitates HIF-1-mediated gene expression. *Genes Dev.*, **14**, 391–396.
46. Ravi,R., Mookerjee,B., Bhujwalla,Z.M., Sutter,C.H., Artemov,D., Zeng,Q., Dillehay,L.E., Madan,A., Semenza,G.L. and Bedi,A. (2000) Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. *Genes Dev.*, **14**, 34–44.

Received June 12, 2006; revised October 13, 2006; accepted October 16, 2006