Int J Clin Exp Med 2016;9(2):1117-1127
www.ijcem.com /ISSN:1940-5901/IJCEM0017993

Original Article

Formononetin suppresses hypoxia inducible factor-1α/inflammatory cytokines expression via inhibiting Akt signal pathway in multiple myeloma cells

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Received October 16, 2015; Accepted January 6, 2016; Epub February 15, 2016; Published February 29, 2016

Abstract: In multiple myeloma (MM), the hypoxic environment is an important factor causing tumor inflammation, which is strongly correlated to disease progression and unfavorable outcome by activating the key transcription factor, hypoxia-inducible factor-1α (HIF-1α). Formononetin is the major active ingredient of Astragalus membranaceus, which has been shown to possess anti-inflammatory effect by in vitro and in vivo study. However, the underlying molecular mechanism of whether formononetin inhibits tumor inflammation remains poorly understood. In this study, we investigated the effects of formononetin on expression of HIF-1α, and its downstream target gene pro-inflammatory cytokines, including TNF-α, TGF-β1, IL-6, and IL-8 in multiple myeloma cell U266. We found that hypoxia induced increase in the level of HIF-1a subunit protein and activated the Akt pathway. Moreover, the treatment with formononetin markedly decreased HIF-1α, TNF-α, TGF-β1, IL-6, and IL-8 expression under hypoxic condition. Mechanistic studies exhibited that formononetin inhibited the production of HIF-1α by reducing phosphorylation of Akt in U266 cells. Furthermore, in vivo study revealed that intragastric administration of formononetin could suppress tumor volumes by anti-inflammation activity. Taken together, our results identify that formononetin suppresses hypoxia-activated pathway that are linked to MM progression, at least partly, by the inhibition of the Akt signaling pathway. Therefore, formononetin may be a new potent therapeutic agent against MM.

Keywords: Formononetin, hypoxia-inducible factor 1 alpha subunit, multiple myeloma, inflammatory cytokines, Akt

Introduction

Multiple myeloma (MM) is one of the most aggressive human cancers and the third leading cause of death worldwide. Despite the recent advance in diagnosis and treatment of MM, it remains a highly lethal disease due to recurrence of metastasis [1]. MM is an example of inflammation-related cancer, as the chronic inflammatory state appears to be necessary for the initiation and development of multiple myeloma. Inflammation, supporting the growth and survival of tumor cells, plays a critical role in the pathophysiology and progression of MM [2]. Hypoxia, a key feature of the tumor microenvironment, has been shown to be a leading cause of inflammation. In the tumor cells, hypoxia-inducible factor-1α (HIF-1α) has been regarded as the most important transcriptional factor promoting inflammatory by up-regulating pro-inflammatory genes cytokines such as tumor necrosis factor-α (TNF-α), transforming growth factor-β1 (TGF-β1), interleukin-6 (IL-6), and IL-8 [3]. These chemokines act on MM cells in an autocrine/paracrine fashion, and enhance MM cell adhesion to stromal cells and metastasis [4].

Under hypoxia, HIF-1α can escape the Von Hippel-Lindau tumor suppressor protein (VHL) binding and proteasomal degradation, translocate to the nucleus, heterodimerize with HIF-1β,

and induce transcription of numerous target genes related to inflammatory [5, 6]. Increased HIF-1α levels are also associated with increased risk of mortality in many human cancers [7]. The previous studies have demonstrated the inflammation microenvironment is hypoxic in MM patients and determined the role of hypoxia in progression and dissemination of MM [8, 9]. These findings suggest HIF-1α could be a potential therapeutic target. Recent studies have shown many mechanisms regulate the HIF-1α expression at the level of transcription, translation and protein stability, such as Akt signaling pathways and prolyl hydroxylase (PHD)-pVHL-dependent mechanisms [10]. Among these mechanisms, serine/threonine kinase (Akt) pathway is an important element in response to hypoxia [11, 12]. Hypoxia can stimulate the activation of Akt pathway and it has become increasingly evident that the increase of HIF-1α synthesis is associated with activated Akt signaling, result in augmenting translation of HIF-1α mRNA into protein [13]. The suppression of Akt can inhibit HIF-1α expression in non-hypoxic and hypoxic cells. Therefore, pharmacological inhibition of HIF-1α activity may represent a useful treatment strategy.

Flavonoids are polyphenolic substances, widely distributed in almost every food plant, that possess antiviral, antimicrobial, anti-inflammatory, anti-thrombotic, antineoplasic, antimutagenic, and cytoprotective effects on different cell types. The dried root of Astragalus membranaceus (Radix Astragali) has a long history of medicinal use in traditional Chinese medicine as an immunomodulating agent in mixed herbal decoctions to treat the diarrhea, common cold, anorexia and fatigue [14]. In contemporary pharmacotherapy, Radix Astragali has been used to ameliorate the side-effects of cytotoxic antineoplastic drugs. Formononetin (**Figure 1A**) is one of the major isoflavonoid constituents isolated from Astragalus membranaceus and has been demonstrated diverse pharmacological benefits. It possesses anti-angiogenic activity in human colon cancer cells and tumor xenograft. Formononetin also promotes cell cycle arrest via downregulation of Akt/Cyclin D1/CDK4 in human prostate cancer cells [15]. In addition, formononetin can anti-inflammatory and antinociceptive through inhibition of TNF-alpha production [16]. However, whether formononetin has an ability to suppress HIF-1α/pro-inflammatory factors expression via inhibition of Akt signaling pathway under hypoxia in MM still remains unclear. Thus, in our study, we investigated the effect of formononetin on HIF-1α/inflammatory agent induction under hypoxia in human MM cell, U266, and its underlying molecular mechanism.

Materials and methods

Cell culture and induction of hypoxia

Multiple myeloma cell, U266 was obtained from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were maintained in Eagle's Minimum Essential Medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Sijiqing, Hangzhou, China) in a humidified atmosphere of 5% CO_2 at 37°C. For hypoxia induction, cells were incubated in a sealed hypoxic chamber flushed with a gas mixture of 94% N_2, 5% CO_2 and 1% O_2.

Plasmid and transfection

A constitutively active mutant D2Akt (T308D/S473D) plasmid was a gift from Peter Vogt (Addgene plasmid # 49192) [17]. Transfection was performed with lipofectamine 2000 following the manufacture's manual.

Cytotoxicity assay

Formononetin (Purity 98%, from Pure-one Bio Technology, CO. LTD, China) was dissolved in DMSO (Sigma-Aldrich), stored at -4°C, and diluted as needed in Eagle's Minimum Essential Medium. Cytotoxic effect of formononetin on proliferating cells was assayed by CCK8 (Dojindo, Kumamoto, Japan). Briefly, cells were seeded onto 96-well plates at a density of 3×10^4 cells/well and treated with various concentrations of formononetin for 8 h. The CCK-8 solution (10 μL) was added to each well and incubated for 3 h. The absorbance was measured at 450 nm by Multiskan MK3 (Thermo Scientific, Shanghai, China). Cell viability was calculated as a percentage of viable cells in the formononetin-treated group versus the untreated control [18].

Lactate dehydrogenase (LDH) toxicity assay

The LDH released into cell cultures is an index of cytotoxicity and evaluation of the permeabil-

ity of cell membrane. U266 were seeded in 96-well plate at a density of 3 × 10^4 cells per well. After incubation with vehicle (0.1% DMSO), 1% Triton X-100 or various concentrations of formononetin for 8 h under hypoxia, cell supernatants were collected and analyzed for LDH activity using LDH cyto-toxicity assay kit from Keygen biotech [19]. The absorbance of formed formazan was read at 490 nm on a microplate reader.

Elisa assay

3 × 10^4 cells were seeded onto 6-well plates in serum-free medium containing various concentrations of formononetin (0, 1, 5 and 10 μM) and incubated for 8 h at hypoxia or normoxia. The conditioned medium was collected, and TNF-α, TGF-β1, IL-6, and IL-8 levels were determined by ELISA kit (Neobioscience, Shenzhen, China) according to the manufacturer's protocols. The content of TNF-α, TGF-β1, IL-6 and IL-8 in tumor tissue were determined by Tissue Elisa Test Kit according to the manufacturer's protocols (Neobioscience, Shenzhen, China) [20].

Quantitative real-time PCR assay

Total RNA was isolated from cells by using RNAiso Plus (TaKaRa, Dalian, China). Reverse transcription was performed with PrimeScript RT reagent kit with gDNA Eraser (TaKaRa), and real time PCR was carried out using SYBR Premix Ex Taq (TaKaRa). The real time PCR reaction contained: 10 μL of SYBR Premix Ex Taq, 0.4 μL of forward primer, 0.4 μL of reverse primer, 0.4 μL ROX Reference Dye, 1 μL of cDNA template, and 7.8 μL of dH_2O. The program was 95°C for 30 s, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The relative expression level of mRNAs was normalized to that of internal control β-actin using the $2^{-\Delta\Delta Ct}$ cycle threshold method. The primer sequences were as follows:

TNF-α (forward) 5'-AGAAGACCCCCCTCGGAACC-3'; TNF-α (reverse) 5'-ATCTGGAGGAAGCGGTAGTG-3'; TGF-β1 (forward) 5'-GGAGAGCAATTCTTACAGGTG-3'; TGF-β1 (reverse) 5'-TAGGAGAAGGAGGGTCTGTC-3'; IL-6 (forward) 5'-CAAGCCTGGGATTATGAAGAAGG-3'; IL-6 (reverse) 5'-AGCACTGGCAGCACAAGGCAAAC-3'; IL-8 (forward) 5'-GGGCCATCAGTTGCAAATC-3'; IL-8 (reverse) 5'-TCCTTCCGGTGGTTTCTTC-3'; HIF-1α (forward) 5'-TGAGGAAATGAGAGAAATGCTTACA-3'; HIF-1α (reverse) 5'-ACACTGAGGTTGGTTACTGTTGGT-3'; GAPDH (forward) 5'-AAAGACCTGTACGCCAACAC-3'; GAPDH (reverse) 5'-GTCATACTCCTGCTTGCTGAT-3'.

Western blot analysis

GSK690693 was purchased from Sigma. Protein extraction from cells and western blot analysis were performed as described previously [21]. Blots were incubated respectively with various appropriately diluted primary antibodies for HIF-1α, TNF-α, TGF-β1, IL-6, IL-8 (Santa Cruz Biotechnology), Akt, and phospho-Akt^{Thr308} (Cell Signaling, Beverly, MA, USA) overnight at 4°C, and then followed by horseradish peroxidase-conjugated goat anti-rabbit or mouse secondary antibody (Santa Cruz Biotechnology) for 2 h at room temperature. The GAPDH (Santa Cruz Biotechnology) was used as the internal control. For quantity, images were analyzed using Image J software.

Mouse xenograft model

Six-week-old male BALB/c nude mice, with body weights of 18-22 g, were procured from Shanghai National Center for Laboratory Animals (Shanghai, China) and maintained in a specific pathogen-free environment. Studies were performed in adherence with the Guidelines established by The Third Xiang-ya Hospital, Central South University, Changsha, China. The mice were subcutaneously injected with 1 × 10^7 U266 cells. Tumor volume was measured every other day with caliper and calculated according to the formula: $V = a^2b/2$, where *a* is the smallest superficial diameter and *b* is the largest superficial diameter. When the tumor volume reached approximately 50 mm^3, the mice (n = 6/group) were randomly assigned to four groups: control group, solvent vehicle group (sodium carboxymethyl cellulose), 20 mg/kg/per/days formononetin, 50 mg/kg/per/days formononetin and cisplatin 5 mg/kg/per/days group. The intragastric administration was done once every day for 25 days. After 25 d, the mice were killed, and the tumors were removed and measured [22].

Immunohistochemistry

Immunohistochemical staining was performed using Ultrasensitive S-P IHC Kit (Maixin, Fuzhou,

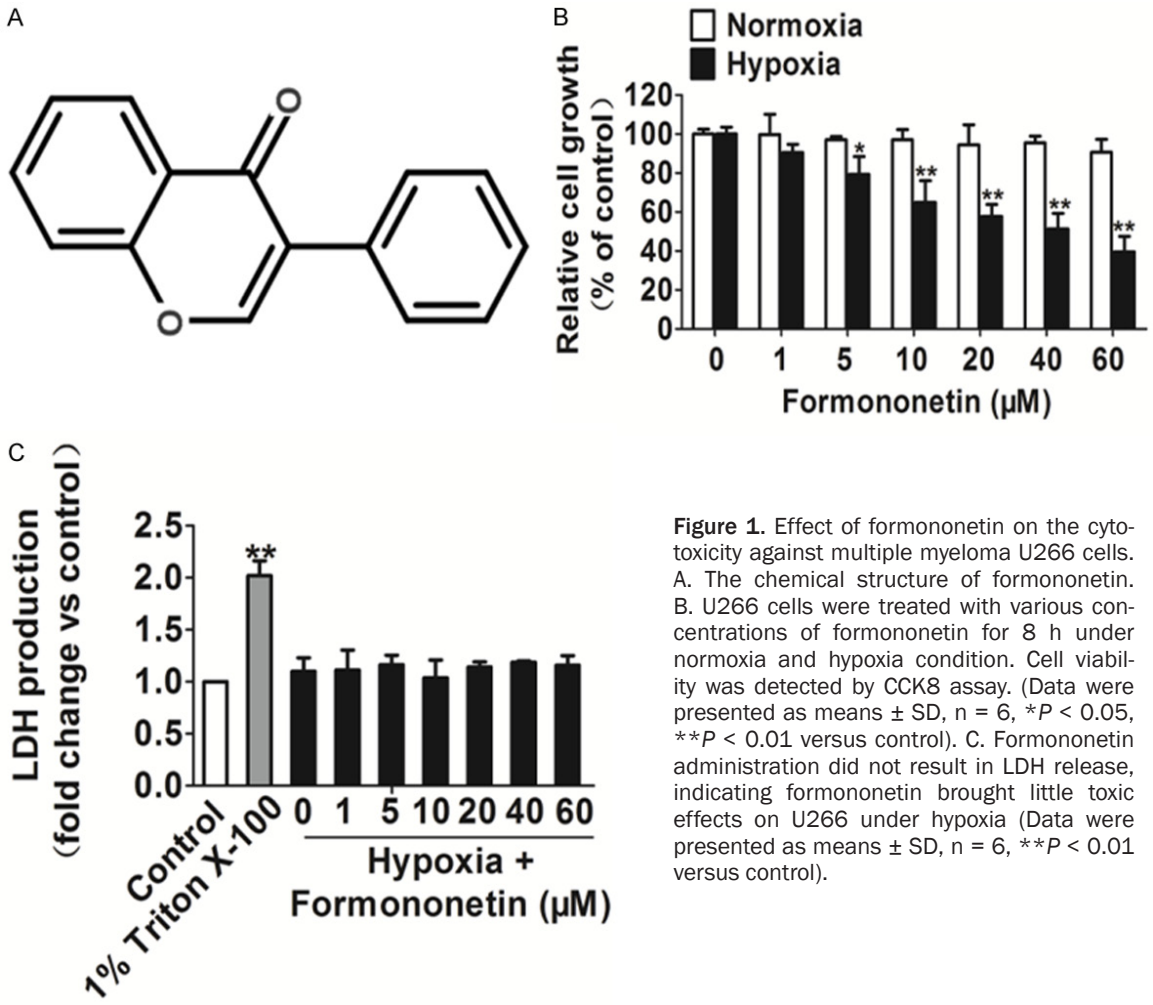

Figure 1. Effect of formononetin on the cytotoxicity against multiple myeloma U266 cells. A. The chemical structure of formononetin. B. U266 cells were treated with various concentrations of formononetin for 8 h under normoxia and hypoxia condition. Cell viability was detected by CCK8 assay. (Data were presented as means ± SD, n = 6, $*P < 0.05$, $**P < 0.01$ versus control). C. Formononetin administration did not result in LDH release, indicating formononetin brought little toxic effects on U266 under hypoxia (Data were presented as means ± SD, n = 6, $**P < 0.01$ versus control).

China) according to the manufacturer's protocols. The sections were incubated with anti-HIF-1α anti-p-Akt^{Thr308} (1:100, Santa Cruz Biotechnology) at 4°C overnight. Then, sections were stained with a streptavidin-peroxidase system, the signal was visualized using diaminobenzidine substrate and counterstaining was done with hematoxylin. For quantity, the microvessel density and the levels of HIF-1α and anti-p-Akt^{Thr308} were measured using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA).

Statistical analysis

All values were expressed as mean ± standard deviation (SD) from triplicate experiments performed in a parallel manner unless otherwise indicated. Data were analyzed using an unpaired, two-tailed Student's t-test. The level of significance was indicated as $*P < 0.05$ and $**P < 0.01$.

Results

Effects of formononetin on growth of multiple myeloma cells U266 in vitro

To determine the potential anti-proliferation effects of formononetin, U266 cells were treated with formononetin under normoxia and hypoxia for 8 h. The results showed that treatment with formononetin at concentrations above 5 μM led to a significant dose-dependent inhibition of U266 cell growth under hypoxia (**Figure 1B**). Besides, formononetin had obscure inhibition effect on the proliferation of U266 under normoxia. To validate whether formononetin would result in toxicity effects on U266, LDH cytotoxicity assay was carried out. As shown in **Figure 1C**, Triton X-100 significantly increased LDH release and formononetin (1~60 μM) brought little toxic effects on U266 under hypoxia for 8 h when compared to formononetin (0 μM).

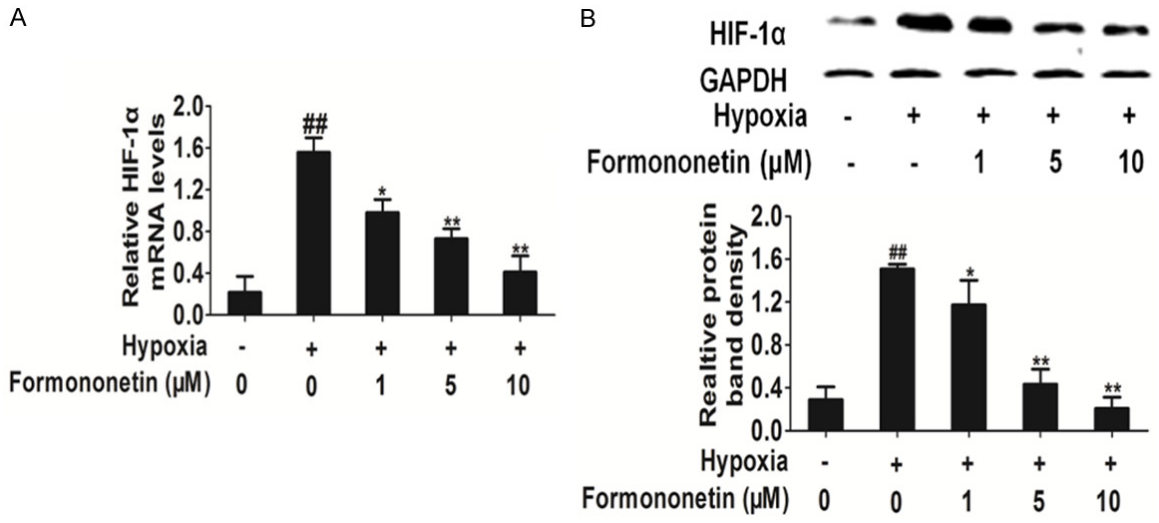

Figure 2. Formononetin attenuated the hypoxia-induced HIF-1α activation in U266 cells. A. Effect of formononetin on hypoxia-inducible factor-1α (HIF-1α) mRNA level under hypoxia. TF-1 cells were treated with various concentrations of formononetin under hypoxia, and HIF-1α mRNA level was detected by real-time PCR. (Data were presented as means ± SD, n = 6, $^{\#\#}P < 0.01$ versus normoxia, $^{**}P < 0.01$ versus 0 μM formononetin under hypoxia). B. Effect of formononetin on HIF-1α protein expression under hypoxia. U266 cells were treated with various concentrations of formononetin for 8 h under hypoxia. HIF-1α protein expression was analyzed by western blots. Bars were the mean ± SD (n = 3). $^{\#\#}P < 0.01$ versus normoxia, $^{*}P < 0.05$, $^{**}P < 0.01$ versus 0 μM formononetin under hypoxia.

Formononetin down-regulates HIF-1α expression in hypoxia

To investigate whether formononetin inhibited HIF-1α expression of U266 cells under hypoxia, real time-PCR and western blots assay were used to evaluate the expression of HIF-1α, the key regulator of hypoxia. The results showed that in hypoxia, the levels of HIF-1α mRNA and protein were significantly inhibited in U266 treated with formononetin (**Figure 2A** and **2B**). Taken together, these findings suggested that formononetin might down-regulate HIF-1α through decreasing translation.

Formononetin suppresses hypoxia-induced inflammatory cytokine expression and secretion in U266 cells

We then examined TNF-α, TGF-β1, IL-6, and IL-8 secretion and expression from the U266 cells under hypoxia and normoxia, as well as the effect of formononetin on that. As shown in (**Figure 3A-D**), TNF-α, TGF-β1, IL-6, and IL-8 were more secreted under hypoxia and formononetin decreased inflammatory cytokines secretion compared to the control group (formononetin 0 μM) under hypoxia. Real time-PCR and western blots were used to measure the expression of TNF-α, TGF-β1, IL-6, and IL-8, the results showed formononetin inhibited hypoxia induced up-regulation of TNF-α, TGF-β1, IL-6, and IL-8 mRNA (**Figure 3E-H**) and protein (**Figure 3I**). All these results indicated that the increases of TNF-α, TGF-β1, IL-6, and IL-8 caused by hypoxia were dramatically inhibited by formononetin in a dose-dependent manner.

The Akt pathway involves hypoxia-induced HIF-1α protein accumulation

It has been reported that Akt signaling pathway may be involved in hypoxia-induced HIF-1α protein accumulation and its downstream target gene expression [14, 15]. To explore whether formononetin can inhibit hypoxia-mediated activation of Akt, U266 cells were treated with 5 μM formononetin for 8 h under hypoxia. Our data showed hypoxia augmented phosphorylation of Akt in U266 cells, which was attenuated by formononetin (**Figure 4A**). To confirm the role of Akt in the HIF-1α/inflammatory cytokines cascade, constitutively active form of Akt (D2Akt) was introduced into U266 and the expression of D2Akt was confirmed by western blot with anti-Flg-tag and anti-Akt antibody (**Figure 4B**). U266 transfected with active Akt could increase TNF-α, TGF-β1, IL-6, IL-8, and HIF-1α expression and inflammatory cytokines secretion under hypoxia (Supplementary Figure 1). As expected, active Akt restored TNF-α, TGF-β1, IL-6, IL-8, and HIF-1α expression in U266

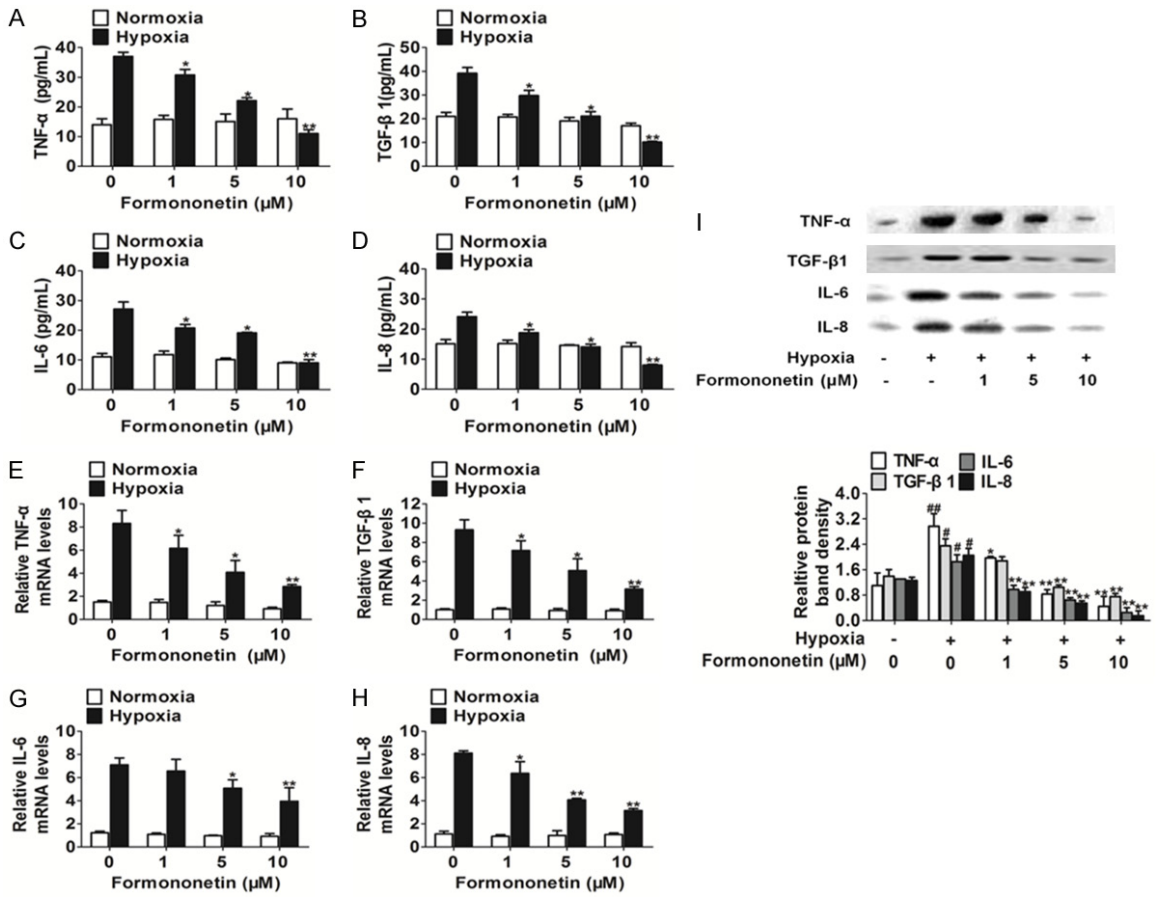

Figure 3. Effect of formononetin on the secretion and expression of inflammatory cytokines in U266 cells under normoxia and hypoxia. U266 cells were treated with formononetin (0, 1, 5, and 10 μM) for 8 h under normoxia and hypoxia. TNF-α (A), TGF-B1 (B), IL-6 (C), AND IL-8 (D) secretion was detected by ELISA assay. Data were presented as means ± SD, n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$ versus 0 μM formononetin under hypoxia. Effect of formononetin on TNF-α (E), TGF-β1 (F), IL-6 (G), and IL-8 (H) mRNA level under normoxia and hypoxia. U266 cells were treated with various concentrations of formononetin (0, 1, 5, and 10 μM) for 8 h under normoxic and hypoxic conditions. TNF-α, TGF-β1, IL-6, and IL-8 mRNA was detected by real-time polymerase chain reaction (PCR) and analyzed by the DDCt method. Data were presented as means ± SD, n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$ versus 0 μM formononetin under hypoxia. (I) Effect of formononetin on inflammatory cytokines protein expression under normoxia and hypoxia. U266 cells were treated with various concentrations of formononetin under normoxic and hypoxic conditions. TNF-α, TGF-β1, IL-6, and IL-8 protein expression was analyzed by western blots. Bars were the mean ± SD (n = 3). $^{\#\#}P < 0.01$ versus normoxia, $^{*}P < 0.05$, $^{**}P < 0.01$ versus 0 μM formononetin.

cells under hypoxia (**Figure 4B**). Consistently, after U266 transfected with active Akt, the secretion of inflammatory cytokines inhibited by formononetin was also abolished (**Figure 4C**).

To further check the role of the Akt signaling pathway in the HIF-1α/inflammatory cytokines cascade, U266 cells were treated respectively with 2 nM GSK690693, a commonly used inhibitor of Akt. The results showed that blocking Akt activation by GSK690693 significantly blunted the elevation of hypoxia induced HIF-1α and inflammatory cytokines (**Figure 4D**). Correspondingly, the increases of inflammatory cytokines and HIF-1α caused by hypoxia were not inhibited by formononetin after blocking Akt pathway (**Figure 4D** and **4E**). In view of these facts, our results indicated that formononetin decreased the hypoxia-induced HIF-1α/inflammatory cytokines protein levels in U266 cells by down-regulation of Akt pathway.

Formononetin inhibits the growth of transplantable tumors

Tumor xenografts transplanted by U266 cells were used to evaluate the antitumor effect of

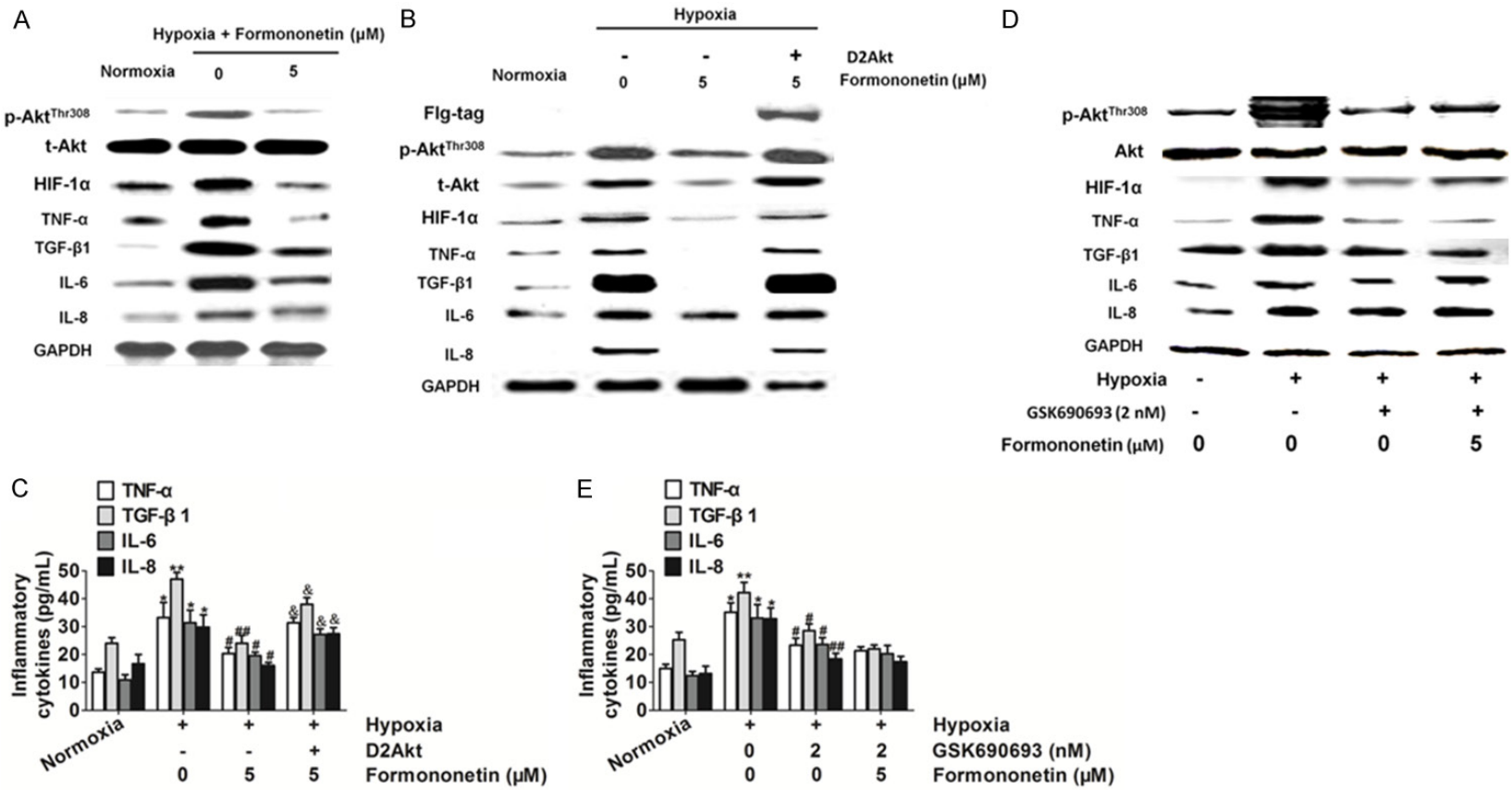

Figure 4. Formononetin inhibited Akt pathway in U266 cells under hypoxia. A. U266 cells were exposed to normoxia or hypoxia in the presence of formononetin (5 μM) for 8 h. Then, the Akt protein expression and its downstream inflammatory cytokines were analyzed by western blots. B. Constitutively active form of Akt (D2Akt) was introduced into cells and expression of D2Akt was confirmed by western blot, and GAPDH was used as loading control. Western blot assay was performed to determine formononetin inhibit HIF-1α/inflammatory cytokines cascade dependent on inactivation of Akt. C. ELISA assay was performed to determine the content of inflammatory cytokines in cells transfected D2Akt plasmid. Data are from three independent experiments and are average ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to normoxia; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to hypoxia; $\&P < 0.05$ compared to formononetin (5 μM) under hypoxia. D. In the presence of GSK690693, protein extracts were analyzed by western blot with antibody against phosphorylated Akt (Thr308) or Akt. Western blot assay was conducted to evaluate the inflammatory cytokines and HIF-1α expression inhibited by formononetin in the presence of GSK690693. E. In the presence of GSK690693 (2 nM), ELISA assay was conducted to evaluate the cell inflammatory cytokines secretion. Data are from three independent experiments and are average ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to normoxia; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to GSK690693 (2 nM) under hypoxia.

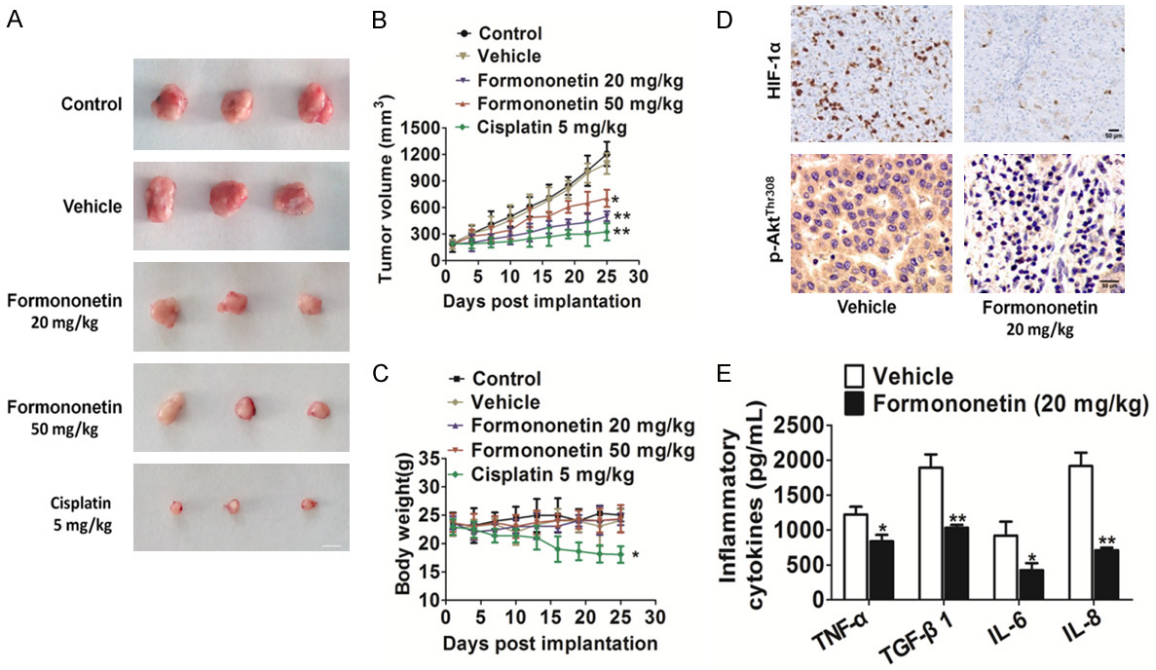

Figure 5. Formononetin inhibited tumor growth in a xenograft mouse model. (A) The BALB/c nude mice were injected with U266 cells for some days followed by treatment with solvent, formononetin or cisplatin for 25 days. Then, the mice were killed, tumor removed and photographed. Scale bar represents 0.5 cm. In addition, the tumor size (B) and body weight (C) were measured. Bars are shown as mean ± SD (n = 6), $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to control. (D) Tumor tissues were prepared for immunohistochemistry detection with antibodies against HIF-1α and p-Akt^{Thr308}. Scale bar represents 50 μm. (E) TNF-α, TGF-β1, IL-6, and IL-8 content from tumor tissue was analyzed by ELISA. The comparisons were made relative to vehicle group, and the different levels of significance were indicated as $^{*}P < 0.05$ and $^{**}P < 0.01$.

formononetin in BALB/c nude mice in vivo. After 25 day treatments, the tumors were moved and photographed (**Figure 5A**). The average tumor size of vehicle group and control group was 1096.9 ± 118.1 mm^3 and 1205.9 ± 138.1 mm^3, while those of formononetin treated groups were 704.1 ± 96 mm^3 (20 mg/kg/days), 501.0 ± 56 mm^3 (50 mg/kg/days) respectively. Cisplatin synergistically suppressed in vivo growth of U266 cells and the average tumor size was 324.1 ± 96 mm^3 (**Figure 5B**). The results indicated that formononetin significantly inhibited tumor growth in a dosage-dependent manner. Moreover, administration of formononetin did not affect the body weight of mice (**Figure 5C**), suggesting the maximal dose of formononetin is not toxic or at least a low toxicity for mice.

To confirm the macroscopic observations and address the potential effect of formononetin in vivo, immunohistochemistry was performed. The results showed that the presence of HIF-1α stained tumor cells in xenografts was dose-dependently reduced by formononetin treatment (**Figure 5D**). The same as HIF-1α, the tumor sections treated with formononetin showed significantly lower levels of p-Akt^{Thr308} than that of vehicle tumor tissue (**Figure 5D**). We examined TNF-α, TGF-β1, IL-6, and IL-8 content in tumor sections, as well as the effect of formononetin on that. As shown in **Figure 5E**, the contents of TNF-α, TGF-β1, IL-6, and IL-8 were inhibited by formononetin compared to the vehicle group. These results further supported that formononetin, a Akt inhibitor, is a potential compound for MM therapy.

Discussion

Multiple myeloma is one of the most common and aggressive human cancers worldwide. Despite the recent advance in diagnosis and treatment of MM, it remains a highly lethal disease due to recurrence of metastasis [1]. MM is an example of inflammation-related cancer and represents a paradigm of the relation occurring between tumor microenvironment

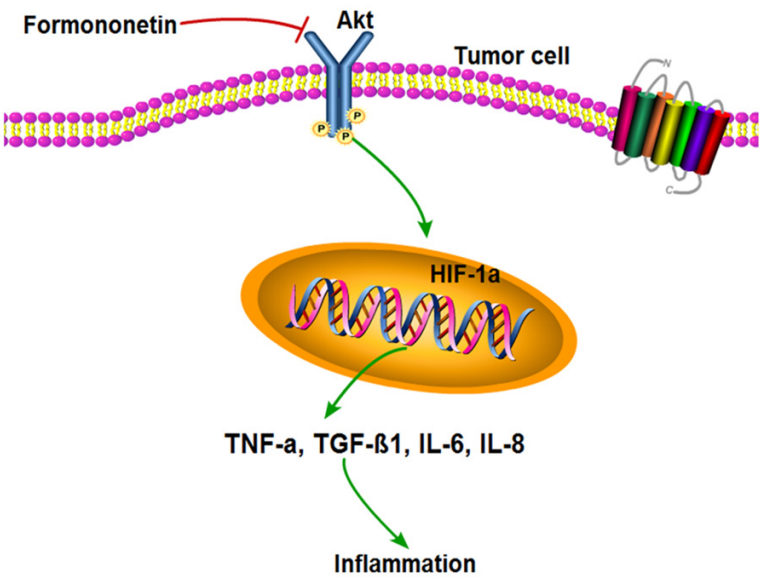

Figure 6. Proposed model by which formononetin treatment suppresses HIF-1α/inflammatory cytokines expression via inhibiting Akt signaling pathway in multiple myeloma cells.

and tumor development. Recent studies have begun to unravel molecular pathways linking inflammation and MM. In the MM microenvironment, smoldering inflammation contributes to proliferation and survival of malignant cells, angiogenesis, metastasis, subversion of adaptive immunity, reduced response chemotherapeutic agents [23]. Hypoxia is a common characteristic of locally advanced solid tumors that has been associated with diminished therapeutic response and, more recently, with inflammation microenvironment. Hypoxia-inducible factor-α plays a critical role in tumor progression and dissemination and it is well established that many malignant tumors express HIF-1α protein in tumor cells [5]. The hypoxic hepatic cancer cells can induce the secretion of cytokines and growth factors, including interleukin-6, members of the super-family of tumor necrosis factor, transforming growth factor β1, tumor necrosis factor, and interleukin-8 [6]. Previous studies have shown that selective HIF-1α inhibition can block inflammation by suppressing the production of pro-inflammatory cytokines, resulting in a potent anti-hepatic cancer effect. Here, our results suggested that the U266 cells augmented the HIF-1α protein accumulation, and the expression and secretion of TNF-α, TGF-β1, IL-6, and IL-8 under hypoxia. Therefore, HIF-1α may be a logical target to control hepatic cancer cell-derived inflammation.

As a promising anti-cancer agent with multiple protein targets, formononetin mediates a wide variety of functional anti-tumor effects including the induction of cell apoptosis, inhibition of proliferation and prevention of cancer metastasis and inflammation [15]. In this experiment, we demonstrated that formononetin significant inhibited U266 cell growth under hypoxia in dose-dependent. In addition, we demonstrated that formononetin dramatically inhibited the expression of HIF-1α protein and mRNA level in a dose-dependent manner in U266 cells under hypoxia, suggesting the suppression of HIF-1α expression by formononetin through decreased protein translation. Hypoxia is a potent inducer of HIF-1α, TNF-α, TGF-β1, IL-6, and IL-8 expression, and this induction can be augmented by Akt activation. In MM, the transcriptional factor HIF-1α promote inflammatory by up-regulating pro-inflammatory genes cytokines. These chemokines act on MM cells in an autocrine/paracrine fashion, and enhance MM progression and deterioration. In vitro ELISA assay, our results indicated that the increases of TNF-α, TGF-β1, IL-6, and IL-8 caused by hypoxia were dramatically inhibited by formononetin in a dose-dependent manner.

Previous studies have proposed that hypoxia can initiate Akt signaling cascade through ligand-independent activation of growth factor receptors [7]. In patients with colorectal cancer and mantle cell lymphoma, hypoxia induced HIF-1α protein accumulation is also in close interaction with an active Akt pathway [24]. In order to assess whether inhibition of HIF-1α by formononetin in hypoxia is involved in Akt pathway, Akt was examined in the presence or absence of formononetin in hypoxia. The phosphorylation of Akt, as well as inflammation mediators were significantly inhibited by formononetin in hypoxic U266 cells. To confirm the role of Akt in formononetin-mediated HIF-1α inhibition, constitutively active form of Akt (D2Akt) was introduced into U266 cells. As expected, active Akt largely restored HIF-1α,

TNF-α, TGF-β1, IL-6, and IL-8 expression in U266 cells under hypoxia. Consistently, Akt over-expression abolishes the inhibit effect of formononetin on TNF-α, TGF-β1, IL-6, and IL-8 secretion. Furthermore, GSK690693, an Akt specific inhibitor was also employed to dissect the role of Akt signaling in HIF-1α and inflammation mediators' expression. Treatment with GSK690693 inhibited hypoxia-stimulated expression of HIF-1α. However, formononetin-mediated inflammation mediators' expression and secretion inhibition was completely blocked by GSK690693. To conclude, these data indicate that suggesting that inhibition of Akt signaling pathway may, at least partly, contribute to the attenuated effect of formononetin on HIF-1α expression and TNF-α, TGF-β1, IL-6, and IL-8 releasing.

A xenograft MM model in nude mice was generated by subcutaneous inoculation of U266 cells to evaluate the anti-tumor activity of formononetin in vivo. As expected, consecutive administration of formononetin for 25 days significantly reduced the tumor size and pro-inflammatory cytokines evidenced by decreased TNF-α, TGF-β1, IL-6, and IL-8 expression in tumors. Furthermore, immunohistochemical staining of the sections revealed decrease of HIF-1α and Akt protein, which was consistent with the findings in vitro. These results demonstrated that formononetin can prevent the development of intratumoral hypoxia that may be attributed to inhibition of tumor inflammation and growth, possibly by attenuating HIF-1α and inflammatory mediator, including TNF-α, TGF-β1, IL-6, and IL-8 expression inside the tumors.

In summary, we conclude that formononetin down-regulates HIF-1α protein levels of U266 cells and inhibits inflammatory mediator content in hypoxia. The inhibition of HIF-1α/inflammatory protein expression is associated with the suppression of Akt pathway under hypoxia, suggesting that formononetin may suppress MM progression and inflammation (**Figure 6**). Taken together, these results not only provide a novel mechanism to explain the anti-inflammation and anti-cancer effects of formononetin, but also imply that formononetin might be a new potential drug for human MM therapy.

Acknowledgements

National Natural Science Foundation of China (H0609).

Disclosure of conflict of interest

None.

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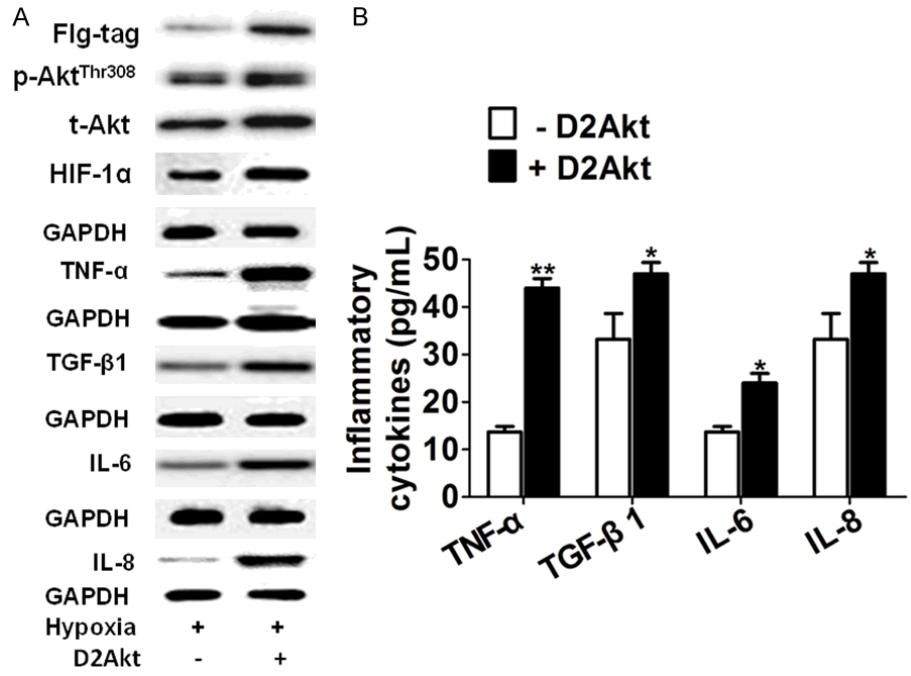

Supplementary Figure 1. Akt over-expression increased inflammatory cytokines and HIF-1α expression under hypoxia. A. Constitutively active form of Akt (D2Akt) was introduced into cells and expression of D2Akt was confirmed by western blot with anti-Flg-tag and anti-Akt antibody. Western blot assay was performed to determine active Akt promoted inflammatory cytokines expression in U266 cells under hypoxia. B. ELISA assay was performed to affirm Akt over-expression increased hypoxia induced inflammatory cytokines secretion in U266 cells.