

Suppressive effect of formononetin on platelet-derived growth factor-BB-stimulated proliferation and migration of vascular smooth muscle cells

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Abstract. Abnormal proliferation and migration of vascular smooth muscle cells (VSMCs) has been implicated in intimal hyperplasia, atherosclerosis and restenosis following percutaneous coronary intervention. Formononetin, a phytoestrogen extracted from the root of *Astragalus membranaceus*, has been widely used in Chinese tradition medicine due to its protective effects against certain symptoms of cancer, hypertension, inflammation, hypoxia-induced cytotoxicity and ovariectomy-induced bone loss. However, the effect of formononetin on platelet-derived growth factor (PDGF)-BB-induced proliferation and migration of VSMCs, as well as the underlying molecular mechanism, remains largely unclear. In the present study, treatment with formononetin significantly inhibited PDGF-BB-induced proliferation and migration of human VSMCs. Investigation into the underlying molecular mechanism revealed that the administration of formononetin suppressed PDGF-BB-stimulated switch of VSMCs to a proliferative phenotype. Furthermore, treatment with formononetin inhibited the PDGF-BB-induced upregulation of cell cycle-related proteins, matrix metalloproteinase (MMP2) and MMP9. In addition, the that administration of formononetin inhibited the phosphorylation of AKT induced by PDGF-BB in VSMCs. The present results suggest that formononetin has a suppressive effect on PDGF-BB-stimulated VSMCs proliferation and migration, which may occur partly via the inhibition of AKT signaling pathway. Therefore, formononetin may be useful for the treatment of intimal hyperplasia, atherosclerosis and restenosis.

Introduction

Under standard physiological conditions, vascular smooth muscle cells (VSMCs) generally remain in a quiescent state. However, in response to vascular damage or inflammatory stimulations, VSMCs may undergo phenotypic changes to an uncontrolled proliferating and migratory state (1). It has been well established that the abnormal proliferation and migration of VSMCs in arterial walls is crucial in the development and progression of cardiovascular disorders, including intimal hyperplasia, arteriosclerosis and restenosis following percutaneous coronary intervention (PCI) (2,3).

Formononetin is an O-methylated isoflavone phytoestrogen extracted from the root of *Astragalus membranaceus*, which has been widely used in Chinese medicine for >2,000 years. *A. membranaceus* has various bioactivities, such as anti-viral, anti-oxidant, anti-tumor, anti-diabetes, anti-inflammation, anti-atherosclerosis, immunomodulation, hepatoprotection, hematopoiesis and neuroprotection (4-7). As an important component of *A. membranaceus*, formononetin has also been demonstrated to have various pharmacological effects. For instance, previous studies have indicated that formononetin is able to inhibit tumor cell proliferation, migration and invasion (8,9), induce tumor cell apoptosis (10), attenuate hydrogen peroxide-induced retinal ganglion cell apoptosis (11), as well as mediate neuroprotection against cerebral ischemia/reperfusion (12).

Recent studies have demonstrated that formononetin may exert protective effects against cardiovascular disorders. Huh *et al* reported that formononetin promoted endothelial repair and wound healing (13). Zhu *et al* found that formononetin had neuroprotective effects against cerebral ischemia and reperfusion injury in rats, and improved cerebrovascular angiogenesis in human umbilical vein endothelial cells (14). However, to the best of our knowledge, the effect of formononetin on VSMCs has not previously been studied.

The aim of the present study was to investigate the effect of formononetin on PDGF-BB-stimulated VSMC proliferation and migration, in addition to elucidating the underlying mechanisms.

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Materials and methods

Materials and agents. Formononetin was purchased from TAOTU Biotech (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM)/F12, fetal bovine serum (FBS), and BCA Protein Assay Kit were purchased from Life Technologies (Carlsbad, CA, USA). An enhanced chemiluminescence kit was purchased from Pierce Biotechnology (Thermo Fisher Scientific, Inc., Rockford, IL, USA). Recombinant human PDGF-BB, dimethyl sulfoxide (DMSO), MTT, bovine serum albumin (BSA) and radioimmunoprecipitation assay (RIPA) buffer were purchased from Sigma-Aldrich (St. Louis, MO, USA). A 24-well chamber was purchased from Corning, Inc., (Corning, NY, USA). Mouse monoclonal antibodies against smoothelin (1:100; ab8969), α -smooth muscle actin (α -SMA; 1:200; ab7817), desmin (1:50; ab8470), cyclin D1 (1:50; ab6152), cyclin-dependent kinase 4 (CDK4; 1:200; ab75511), matrix metalloproteinase 2 (MMP2; 1:100; ab86607), MMP9 (1:100; ab58803), phospho-AKT, mouse AKT (1:100; ab105731) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:50; ab8245), in addition to goat anti-mouse secondary antibody were obtained from Abcam (1:20,000; ab6785; Cambridge, MA, USA).

Cell culture. Human dermis VSMCs were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). VSMCs were cultured in DMEM/F12 medium with 10% FBS at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

MTT assay. An MTT assay was performed for examining the cell proliferation. Three groups were established, as follows: Control group, VSMCs were without any treatment; PDGF-BB group, VSMCs were treated with PDGF-BB (30 ng/ml); and PDGF-BB + formononetin group, VSMCs were treated with PDGF-BB (30 ng/ml) and formononetin (1 μ M). Cells (5×10^3) in each group were seeded into 96-well plates, and cultured for 0, 12, 24 and 48 h, respectively. Then, 10 μ l MTT (10 mg/ml) was added to the cells and incubated for 4 h prior to termination of the reaction by removing the supernatant and adding 100 μ l DMSO to dissolve the formazan product. Following incubation for 30 min, the optical density of each well was measured at 570 nm using a plate reader (ELx808; BioTek Instruments, Inc., Winooski, VT, USA).

Scratch assay. Cell migration in each group was examined using a costar 24-well chamber (Corning Inc., Shanghai, China). The cells were counted under a CX23 microscope (Olympus, Tokyo, Japan). In brief, cell suspension (5×10^5 cells/ml) was prepared in DMEM/F12 medium. In accordance with the manufacturer's instructions, 500 μ l DMEM/F12 with 10% FBS was added to the lower chamber, and 300 μ l cell suspension was added into the upper chamber. In the PDGF-BB group, PDGF-BB (30 ng/ml) was also added to the lower wells. In the PDGF-BB + formononetin group, the lower wells contained PDGF-BB (30 ng/ml) and formononetin (1 μ M). The relative migration in the PDGF-BB group is presented as the cell number ratio of PDGF-BB versus the control. Similarly, the relative migration in the PDGF-BB+formononetin group is presented as the cell number ratio of PDGF-BB+formononetin versus the control. After 24 h incubation at 37°C with 5%

CO₂, cells that had not migrated through the membrane were removed, while cells that had been stained with crystal violet dye (Beyotime Institute of Biotechnology, Haimen, China) for 30 min, then rinsed with water and dried in air. The stained cells were counted and the relative cell migration was determined.

Western blot analysis. Cells were washed with phosphate-buffered saline once and 500 μ l RIPA buffer was added to lyse the cells. Cells were then centrifuged at 8,000 \times g for 10 min at 4°C, and the supernatant containing the protein was collected. The concentration of protein was determined using a BCA Protein Assay kit, in accordance with the manufacturer's instructions. Next, 50 μ g protein was run on a 12% SDS-PAGE gel (Beyotime Institute of Biotechnology) and blotted onto polyvinylidene difluoride membranes (Thermo Fisher Scientific, Inc.), which were blocked in 5% BSA for 1.5 h at room temperature, followed by incubation overnight at 4°C with the indicated antibodies. The membranes were rinsed and incubated for 1 h at room temperature with the appropriate peroxidase-conjugated secondary antibodies. Chemiluminescent detection was performed using the enhanced chemiluminescence kit. The relative protein expression was analyzed by Image Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) and presented as the density ratio of FSCN1 versus GAPDH.

Statistical analysis. Data is presented as the mean \pm standard deviation of at least three independent experiments. SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. One-way analysis of variance was used to analyze the differences between groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Formononetin inhibited PDGF-BB-stimulated proliferation and migration of VSMCs. The effects of formononetin on PDGF-BB-induced VSMCs proliferation and migration were investigated first. MTT assay data showed that the PDGF-BB treatment enhanced the proliferation of VSMCs compared with the control group, which was notably attenuated by the treatment with formononetin (Fig. 1A). These data suggest that formononetin inhibits PDGF-BB-stimulated VSMCs proliferation. Subsequently, the effect of formononetin on the PDGF-BB-stimulated migration of VSMCs was investigated. The results of a scratch assay showed that treatment with PDGF-BB significantly promoted VSMCs migration compared with the control group; however, formononetin significantly attenuated the upregulation of PDGF-BB-induced VSMC migration (Fig. 1B), suggesting that formononetin has an inhibitory effect on PDGF-BB-induced VSMCs migration.

Formononetin inhibited the PDGF-BB-induced phenotype change of VSMCs. Under normal physiological conditions, vascular smooth muscle cells (VSMCs) generally remain in a quiescent state. However, in response to vascular damage or inflammatory stimulation, VSMCs may undergo phenotypic changes to an uncontrolled proliferating and migratory state (1). Smoothelin, α -SMA and desmin are markers for the

quiescent phenotype of VSMCs (15). Therefore, the expression levels of these proteins were detected in each group. As shown in Fig. 2, administration of PDGF-BB significantly inhibited the protein expression levels of smoothelin, α -SMA and desmin in VSMCs, which cause VSMCs to dedifferentiate into a proliferative phenotype. However, treatment with formononetin significantly attenuated the PDGF-BB-induced downregulation of α -SMA, smoothelin and desmin protein expression in VSMCs (Fig. 2), suggesting that formononetin has an inhibitory effect on PDGF-BB-induced phenotype switch in VSMCs.

Formononetin inhibited the PDGF-BB-induced expression of cell cycle-related proteins in VSMCs. Cell cycle-related proteins, including CDK2, CDK4, cyclin D1 and cyclin E, are crucially involved in the regulation of cell cycle progression, as well as cell proliferation (16). It has been reported that formononetin has effects on the expression of cell cycle-related proteins (8). Therefore, the effect of formononetin on VSMCs proliferation may be associated with the expression levels of cell cycle-related proteins. Western blot analysis was conducted to determine the protein levels of CDK2, CDK4, cyclin D1, and cyclin E in each group. As shown in Fig. 3, administration of PDGF-BB significantly enhanced the protein expression levels of CDK2, CDK4, cyclin D1, and cyclin E in VSMCs; however, treatment with formononetin significantly suppressed PDGF-BB-stimulated upregulation of CDK2, CDK4, cyclin D1 and cyclin E, suggesting that the suppressive effect of formononetin on PDGF-BB-induced VSMCs proliferation may partly occur via the inhibition of the expression of cell cycle-related proteins.

Formononetin suppressed PDGF-BB-induced upregulation of MMP2 and MMP9 in VSMCs. It has been well established that MMP2 and MMP9 play key roles in the regulation of cell migration (9). Therefore, the protein expression levels of MMP2 and MMP9 were determined in the VSMCs in each group. As shown in Fig. 4, MMP2 and MMP9 were significantly upregulated following treatment with PDGF-BB, which was significantly attenuated by treatment with formononetin. These results suggest that the suppressive effect of formononetin on PDGF-BB-induced VSMCs migration is mediated by the inhibition of MMP2 and MMP9 protein expression.

Formononetin suppressed PDGF-BB-induced activation of AKT signaling in VSMCs. AKT signaling pathway has been implicated in the regulation of cell proliferation and migration, in addition the expression of cell cycle-related proteins and MMPs (8,17). Accordingly, the activity of AKT signaling in VSMCs was evaluated in the present study. As shown in Fig. 5, the phospho-AKT protein expression was significantly upregulated by treatment with PDGF-BB, when compared with the control group, suggesting that PDGF-BB is able to activate the AKT signaling pathway. However, treatment with formononetin effectively suppressed PDGF-BB-stimulated upregulation of phospho-AKT protein level in VSMCs, suggesting that formononetin is able to inhibit PDGF-BB-induced activation of AKT signaling in VSMCs.

Discussion

The results of the present study suggest that formononetin exerted an inhibitory effect against PDGF-BB-stimulated VSMCs proliferation and migration. Formononetin was able to inhibit the PDGF-BB-stimulated change of VSMCs into a proliferative phenotype, suppressed the enhanced expression of cell cycle-related proteins and MMPs, in addition to down-regulating the activity of AKT signaling.

VSMCs are continually stimulated by the biochemical components in the blood compartment, which may affect their phenotypes such as cell proliferation and migration. Thus VSMCs are involved in the physiological and pathological processes in the vascular wall (18,19). For example, following vascular injury various cytokines, including PDGF-BB, are released by endothelial cells and macrophages. These stimulate the abnormal proliferation and migration VSMCs, a key promoter in the initiation of intimal hyperplasia, which can further lead to arteriosclerosis and restenosis following PCI (20-22). Therefore, inhibition of PDGF-BB-induced VSMCs proliferation and migration is crucial for the prevention of atherosclerosis and restenosis. Formononetin has been shown to inhibit the proliferation of multiple types of cancer cells. Li *et al* showed that formononetin inhibited the proliferation of human prostate cancer cells via inducing cell cycle arrest (8). Liu *et al* showed that formononetin suppressed proliferation while inducing the apoptosis of osteosarcoma cells (23). However, the effects of formononetin on VSMCs proliferation have been hitherto unclear. Herein, it was reported that formononetin suppressed PDGF-BB-stimulated VSMCs proliferation. Furthermore, the results indicate that PDGF-BB could induce VSMCs to dedifferentiate into a proliferative phenotype, as suggested by the downregulation of SMA, smoothelin and desmin, which is consistent with previous studies (16,24). However, treatment with formononetin attenuated the PDGF-BB-induced downregulation of SMA, smoothelin and desmin, indicating that formononetin inhibited the PDGF-BB-induced phenotype change in VSMCs.

Cell cycle-related proteins such as cyclin D1, cyclin E, CDK2 and CDK4 are crucially involved in the regulation of cell proliferation. Previous studies have shown that these cell cycle-related proteins are associated with PDGF-BB-stimulated VSMCs proliferation (25,26). Furthermore, it has been reported that formononetin is able to mediate the expression of these proteins. For example, formononetin promotes cell cycle arrest via the downregulation of cyclin D1 and CDK4 expression in human prostate cancer cells (8). The present results suggest that treatment with formononetin significantly attenuated the PDGF-BB-stimulated upregulation of cyclin D1, cyclin E, CDK2 and CDK4.

MMP2 and MMP9 have been shown to play key roles in the regulation of VSMCs migration. For instance, Ding *et al* observed that resistin stimulated MMP-2 and MMP-9 expression and VSMC migration, while neutralizing antibodies against MMP-2 and MMP-9 effectively reversed resistin-stimulated VSMC migration (27). In addition, MMP2 and MMP9 are involved in intimal hyperplasia. Guo *et al* found that neointimal hyperplasia was reduced in MMP9^{-/-} or MMP2^{-/-} mice after femoral artery injury (28). In the present study, it was shown that treatment of PDGF-BB increased the expression levels

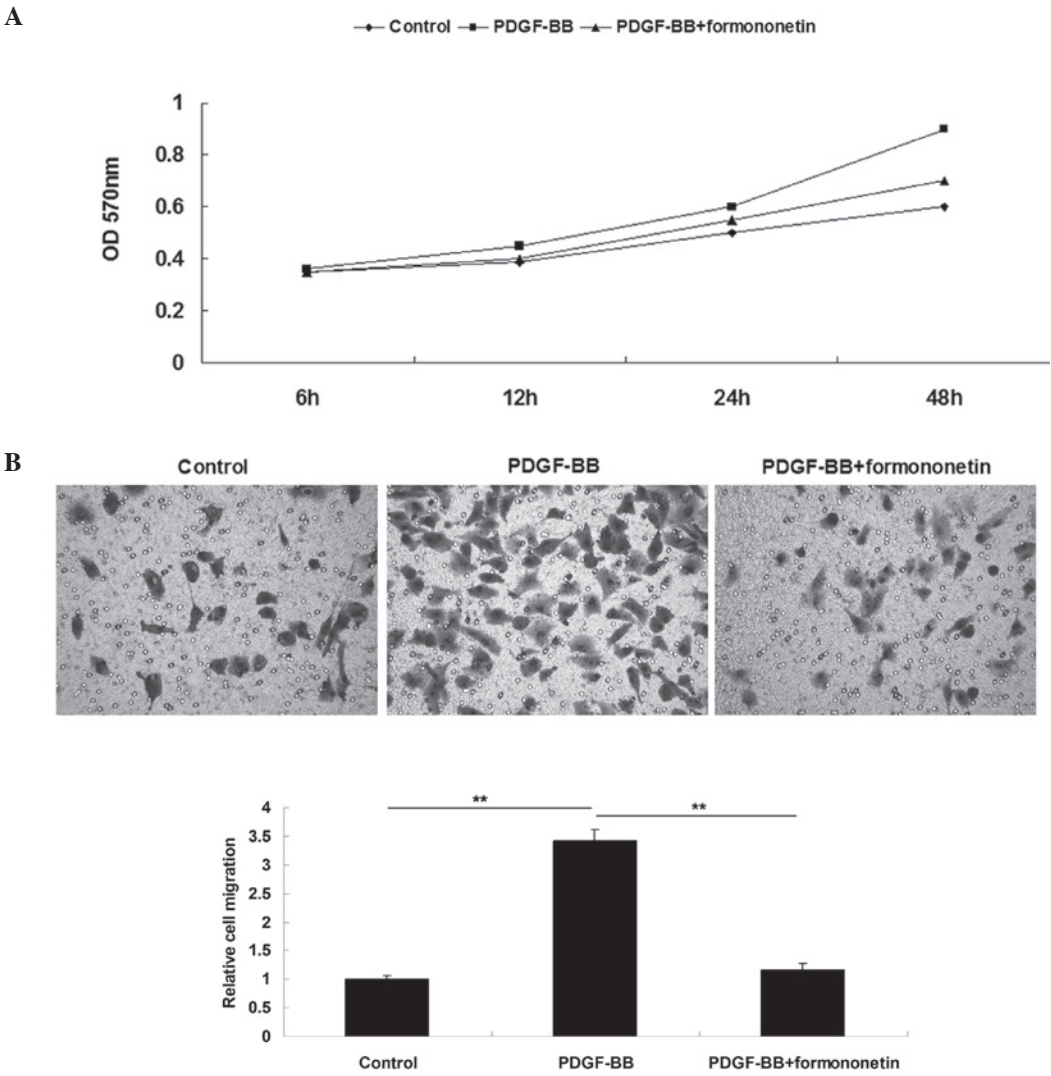


Figure 1. Formononetin inhibited PDGF-BB-induced proliferation and migration of vascular smooth muscle cells (VSMCs). (A) MTT assay was performed to determine VSMC proliferation. (B) Transwell assay was performed to determine VSMC migration. **P<0.01. One way analysis of variance was used to analyze the differences between groups. OD, optical density; PDGF-BB, platelet-derived growth factor-BB.

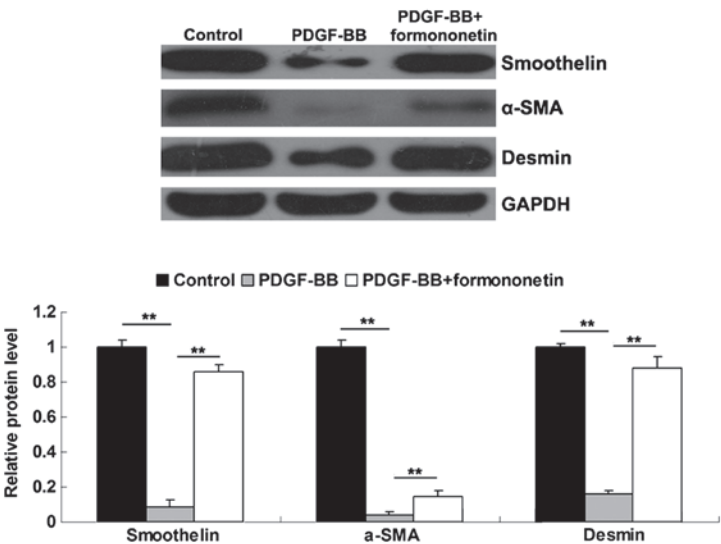


Figure 2. Formononetin inhibited PDGF-BB-induced phenotype switch of vascular smooth muscle cells. Western blot analysis was conducted to evaluate the protein expression of smoothelin, α-SMA and desmin. GAPDH was used as an internal reference. **P<0.01. One way analysis of variance was used to analyze the differences between groups. PDGF-BB, platelet-derived growth factor-BB; α-SMA, α-smooth muscle actin.

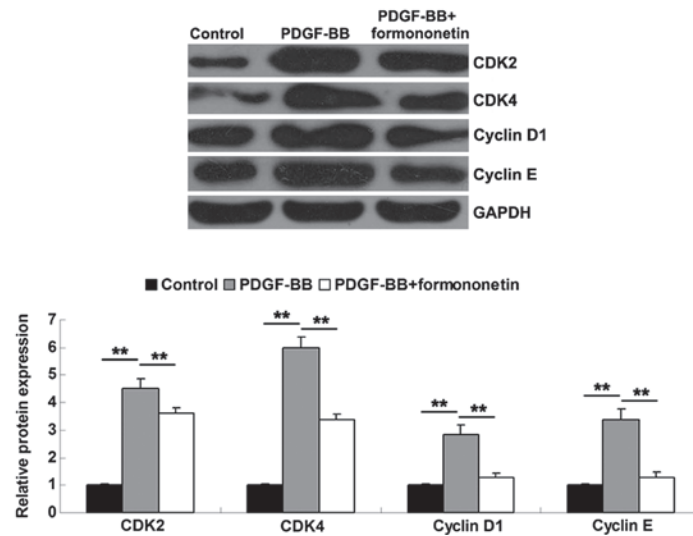


Figure 3. Formononetin suppressed PDGF-BB-induced upregulation of cell cycle-related proteins in vascular smooth muscle cells. Western blot analysis was performed to determine the expression levels of CDK2, CDK4, cyclin D1 and cyclin E. GAPDH was used as an internal reference. **P<0.01. One way analysis of variance was used to analyze the differences between groups. PDGF-BB, platelet-derived growth factor-BB; CDK, cyclin-dependent kinase.

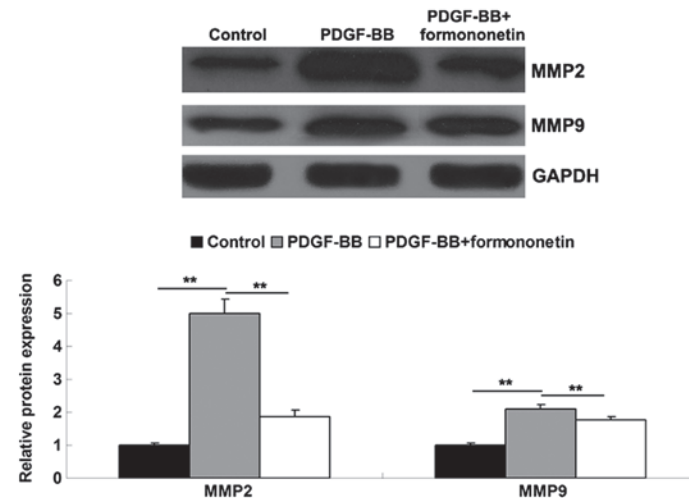


Figure 4. Formononetin inhibited PDGF-BB-induced upregulation of MMP2 and MMP9 in vascular smooth muscle cells. Western blot analysis was performed to determine the protein expression levels of MMP2 and MMP9. GAPDH was used as an internal reference. **P<0.01. One way analysis of variance was used to analyze the differences between groups. PDGF-BB, platelet-derived growth factor-BB; MMP, matrix metalloproteinase.

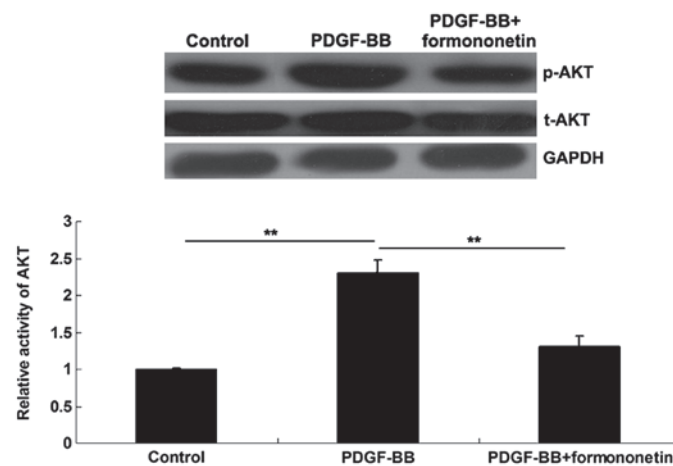


Figure 5. Formononetin suppressed PDGF-BB-induced activation of AKT signaling in VSMCs. Western blot analysis was performed to determine the protein expression of p-AKT and t-AKT in each group. GAPDH was used as an internal reference. **P<0.01. One way analysis of variance was used to analyze the differences between groups. PDGF-BB, platelet-derived growth factor-BB; p-AKT, phosphorylated AKT; t-AKT, total-AKT.

of MMP2 and MMP9, which is consistent with the findings of Guo *et al* (28). Moreover, treatment with formononetin markedly suppressed the PDGF-BB-stimulated upregulation of MMP2 and MMP9, suggesting that the suppressive effect of formononetin on PDGF-BB-stimulated VSMCs migration is partly mediated via the inhibition of MMP2 and MMP9 expression.

AKT signaling has been implicated in various cellular biological processes, such as cell survival, apoptosis, cell cycle progression and angiogenesis, in addition to cell migration and invasion (29,30). In addition, it has been demonstrated that AKT signaling is involved in regulating the expression of a number of cell cycle-related proteins and MMPs (9,31,32). Therefore, the activity of AKT signaling after treatment with PDGF-BB with or without formononetin was evaluated in the present study. Treatment with PDGF-BB appeared to enhance the phosphorylated protein level of AKT, indicating that the activity of AKT signaling was upregulated, which is consistent with previous studies (15,33,34). However, treatment with formononetin effectively suppressed PDGF-BB-stimulated upregulation of phospho-AKT protein level in VSMCs, suggesting that formononetin is able to inhibit PDGF-BB-induced activation of AKT signaling in VSMCs.

In conclusion, the results of the present study demonstrate that treatment with formononetin is able to inhibit PDGF-BB-induced VSMC proliferation and migration via the inhibition of phenotype switch, expression of cell cycle-related proteins and MMPs, in addition to the activity of AKT signaling. Therefore, formononetin may require further investigation as a potential treatment for intimal hyperplasia, atherosclerosis and restenosis following PCI.

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