Crocin prevents platelet-derived growth factor BB-induced vascular smooth muscle cells proliferation and phenotypic switch

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Received December 31, 2017; Accepted March 29, 2018

DOI: 10.3892/mmr.2018.8854

Abstract. The phenotypic switch of vascular smooth muscle cells (VSMCs) is a major initiating factor for atherosclerotic cardiovascular diseases. Platelet-derived growth factor-BB (PDGF-BB) initiates a number of biological processes that contribute to VSMC proliferation and phenotypic switch. Crocin, a component of saffron, has been reported to inhibit atheromatous plaque formation. However, the effects of crocin on PDGF-BB-induced VSMC proliferation and phenotypic switch remain unclear. The aim of the present study was to investigate the role of crocin on PDGF-BB-induced VSMCs proliferation and phenotypic switch and its underlying mechanisms. Cell proliferation and markers of VSMCs phenotypic switch were measured using a Cell Counting Kit-8 assay and western blot analysis, respectively. The signaling pathways involved in the effects of crocin on VSMCs were validated by western blot analysis with or without the use of specific pathway inhibitors. Crocin significantly inhibited PDGF-BB-induced VSMCs proliferation compared with the PDGF-BB only group ($P<0.05$). In addition, crocin significantly abrogated the PDGF-BB-induced increase in contractile protein α-smooth muscle actin, calponin and decrease in synthetic proteins osteopontin (OPN) in a concentration dependent manner ($P<0.05$). In addition, crocin slowed PDGF-BB-induced Janus kinase (JAK)-signal transducer and activator of transcription 3 (STAT3) and extracellular signal-regulated kinase (ERK)/Kruppel-like factor 4 (KLF4) signaling activation in VSMCs. By applying the JAK inhibitor (AG490) and ERK1/2 inhibitor (U0126), the results suggested that the crocin inhibited PDGF-BB-induced VSMCs phenotypic switch through the JAK/STAT3 and ERK/KLF4 signaling pathways. These results suggested that crocin may effectively prevent PDGF-BB-induced VSMCs proliferation and phenotypic switch and may be a promising candidate for the therapy of atherosclerotic cardiovascular diseases.

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Abbreviations: VSMCs, vascular smooth muscle cells; PDGF-BB, platelet-derived growth factor-BB; α-SMA, α-smooth muscle actin; OPN, osteopontin; STAT3, signal transducers and activators of transcription; ERK1/2, extracellular signal-regulated kinase 1/2; KLF4, kruppel-like factor 4

Key words: vascular smooth muscle cells, platelet-derived growth factor-BB, proliferation, phenotypic switch, crocin

Introduction

Vascular smooth muscle cells (VSMCs) play an important role in blood vessel tone regulation and in vascular growth and response to injuries (1). In the media layer of mature blood vessels, VSMCs exhibit extensive plasticity and can go through phenotypic modulation from a quiescent contractile state to a proliferative synthetic state in response to various environmental stimuli, such as growth factors, reactive oxidative species, and mechanical injury (2). It is noteworthy that among abovementioned stimuli, platelet-derived growth factor-BB (PDGF-BB) modulated phenotypic switch has been thoroughly established and subsequently leads to the formation of neointima in response to vascular injury (3,4). This phenotypic modulation is characterized by the alteration of proliferation and alterations in the expression of phenotypic markers, such as alpha smooth muscle actin (α-SMA), calponin and osteopontin (OPN) (5). Prevention of PDGF-BB-induced VSMC phenotypic switch and proliferation leading to the attenuated intimal hyperplasia and vascular remodeling (6). On this basis, deeper understanding of the mechanisms that may control VSMCs phenotype modulation could be a critical therapeutic target in treatment of atherosclerotic cardiovascular diseases.

Crocin is one of the major biologically active substances of saffron. The well-known pharmacological effects of crocin are anti-oxidant (7), anti-cancer (8) and neuroprotective activities (9). Previously, crocin was shown with the ability to significantly inhibit atheromatous plaque formation in atherosclerotic quails and its mechanisms may through decreasing the EC apoptosis and inhibiting the elevated calcium ion in oxidatively modified low-density lipoprotein induced VSMCs (10). Recently, a study found crocin could decrease blood lipid levels and inhibit lipogenesis by suppressing the expression of lipogenesis-related proteins and elevating lipid catabolism-related proteins (11). Moreover, crocin could alleviate the inflammation in a VD3-induced rat coronary atherosclerosis model by inhibiting NF-κB p65 nuclear

translocation (11). These all suggested the potential protective effects of crocin in the initiation and progression of atherosclerosis. Nevertheless, to date, whether crocin could regulate VSMC phenotypic switch still needs to be elucidated.

In the present study, we aimed to explore the role of crocin in PDGF-BB-mediated phenotype switching of VSMCs. Primary rats aortic VSMCs were treated with PDGF-BB followed by various concentration of crocin. The proliferative rates and phenotypic switch of VSMCs were then measured. Subsequently, the potent mechanisms of the action were investigated.

Materials and methods

Cell and reagents. VSMCs were isolated from thoracic aortas of male SD rats as previous indicated (12). Animal studies conformed to the ARRIVE guidelines (2013) and were approved by the Institutional Animal Care and Use Committee of the First Hospital of China Medical University. The culture medium was Dulbecco's modified Eagle's media (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 100 U/ml penicillin/100 μg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.), containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Cells were maintained at 37˚C and 5% CO_2. VSMCs of passages 5-9 were used for experiments. Crocin (cat. no. 42553-65-1, purity >97%, structure presented in Fig. 1A) and PDGF-BB were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against α-SMA, OPN, calponin and kruppel-like factor 4 (KLF4) were obtained from Abcam (Cambridge, MA, USA). Antibodies against phosphorylated (p-)JAK1, JAK1, p-JAK2, JAK2, signal transducers and activators of transcription (p-STAT3), STAT3, extracellular signal-regulated kinase (p-ERK1/2), and ERK1/2 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against GAPDH was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). AG490 and U0126 were acquired from MedChem Express (Monmouth Junction, NJ, USA). The concentration of inhibitors was selected according to previous studies (13,14).

Cell treatment. After an initial 24 h of culture in serum-free medium, VSMCs were exposed to 20 ng/ml PDGF-BB for 24 h with or without various concentration of crocin (10, 50, 100 μM). For pathways inhibitors, VSMCs were pre-treatment for 1 h with AG490 or U0126 at the final concentration of 10 and 50 μM, respectively.

Cell viability assay. VSMCs were cultured in 96-well plates ($5x10^3$ cells/well). After reaching a confluence of 85%, cells were treated with different concentration of crocin, and stimulated with 20 ng/ml PDGF-BB for 24 h. Then, 10 μl Cell Counting Kit-8 (CCK-8) reagent was added to each well followed by incubation for an additional 2 h at 37˚C. The absorbance of cells was measured at 450 nm using a microplate reader.

Immunofluorescence staining. Following treatment of VSMCs with PDGF and crocin, cells were fixed in 4% paraformaldehyde for 15 min and permeabilized in 0.1% Triton X-100 for another 15 min at room temperature. Subsequently, cells were blocked with 2% BSA for 30 min at room temperature followed by incubation with α-SMA antibody (1:400) at 4˚C overnight. After washes with PBS for three times, cells were incubated with goat anti-rabbit IgG H&L Alexa Fluor® 488 (1:500) at room temperature for another 1 h. Finally, nuclei were stained with DAPI for 5 min. Images were taken under a fluorescence microscope (IX73-A12FL/PH; Olympus, Tokyo, Japan) at 200x magnification.

Western blot analysis. After treatment, the cells were lysed in RIPA lysis buffer supplemented with protease/phosphatase inhibitor for 15 min at 4˚C and total protein concentrations were measured using the BCA assay. Protein samples were loaded on 10-12% SDS-PAGE and transferred to PVDF membranes followed by blocked with 5% skim milk in TBST at room temperature for 1 h. After washing, the membranes were incubated with the primary antibodies against α-SMA (1:1,000), calponin (1:1,000), OPN (1:500), p-JAK1 (1:1,000), JAK1 (1:1,000), p-JAK2 (1:1,000), JAK2 (1:1,000), p-STAT3 (1:1,000), STAT3 (1:1,000), KLF4 (1:1,000), p-ERK1/2 (1:10,000), ERK1/2 (1:10,000) and GAPDH (1:1,000) overnight at 4˚C. After three washes with TBST, the blots were incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit antibody/mouse antibodies at room temperature for 1 h. Signals were visualized using an enhanced chemiluminescence kit and densitometry analysis of the immunoblots was carried out using Image J software (National Institute of Health, Bethesda, MD, USA).

Data analysis. All data are expressed as mean ± SD and analyzed using SPSS version 19.0 (SPSS, Inc., Chicago, IL, USA). Statistical analyses were performed using one-way ANOVA with Tukey's post hoc test. The value of $P<0.05$ was statistically significant.

Results

Crocin inhibits PDGF-BB-induced VSMCs proliferation and phenotypic switch. First, we assessed the effect of crocin on VSMCs proliferation. As shown in Fig. 1B, treatment with different concentrations (10, 50, and 100 μM) of crocin for 24 or 48 h did not affect the proliferation of the VSMCs, as detected by CCK-8 assay. Then, we investigated the effects of crocin on PDGF-BB (20 ng/ml) treated VSMCs. The results in Fig. 2A showed that cell proliferation rate was dramatically increased in PDGF-BB treated VSMCs and was decreased with additional treatment with crocin in a concentration dependent manner. We hypothesized that the increased VSMCs proliferation rate may largely due to the transition from a contractile phenotype to a synthetic phenotype. Therefore, the markers of VSMCs phenotypic switch were tested under crocin treatment. Indicated by western blot analysis, PDGF-BB treatment significantly decreased the expression levels of α-SMA and calponin, whereas increased the expression levels of OPN. Intriguing, these alterations were reversed by crocin in a concentration-dependent manner (Fig. 2B-E). Moreover, the results of immunofluorescent staining with α-SMA revealed that compared with control group, PDGF-BB not only reduced the fluorescence intensity of α-SMA, but also perturbed myofibrillar arrangement in the cytoplasm. After crocin treatment, above changes were gradually abolished (Fig. 2F).

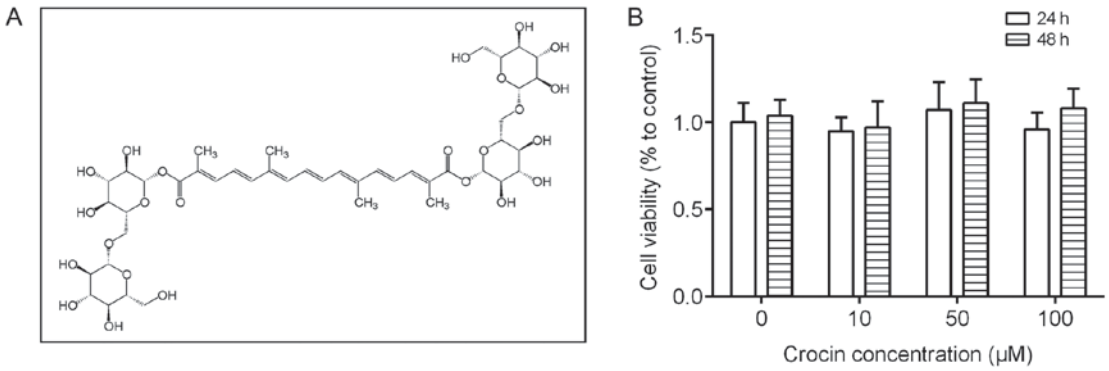

Figure 1. (A) Chemical structure of crocin. (B) Cell Counting Kit-8 assay was performed to determine vascular smooth muscle cells proliferation rate with the treatment of different concentration of crocin (0, 10, 50, and 100 μM) at 24 and 48 h.

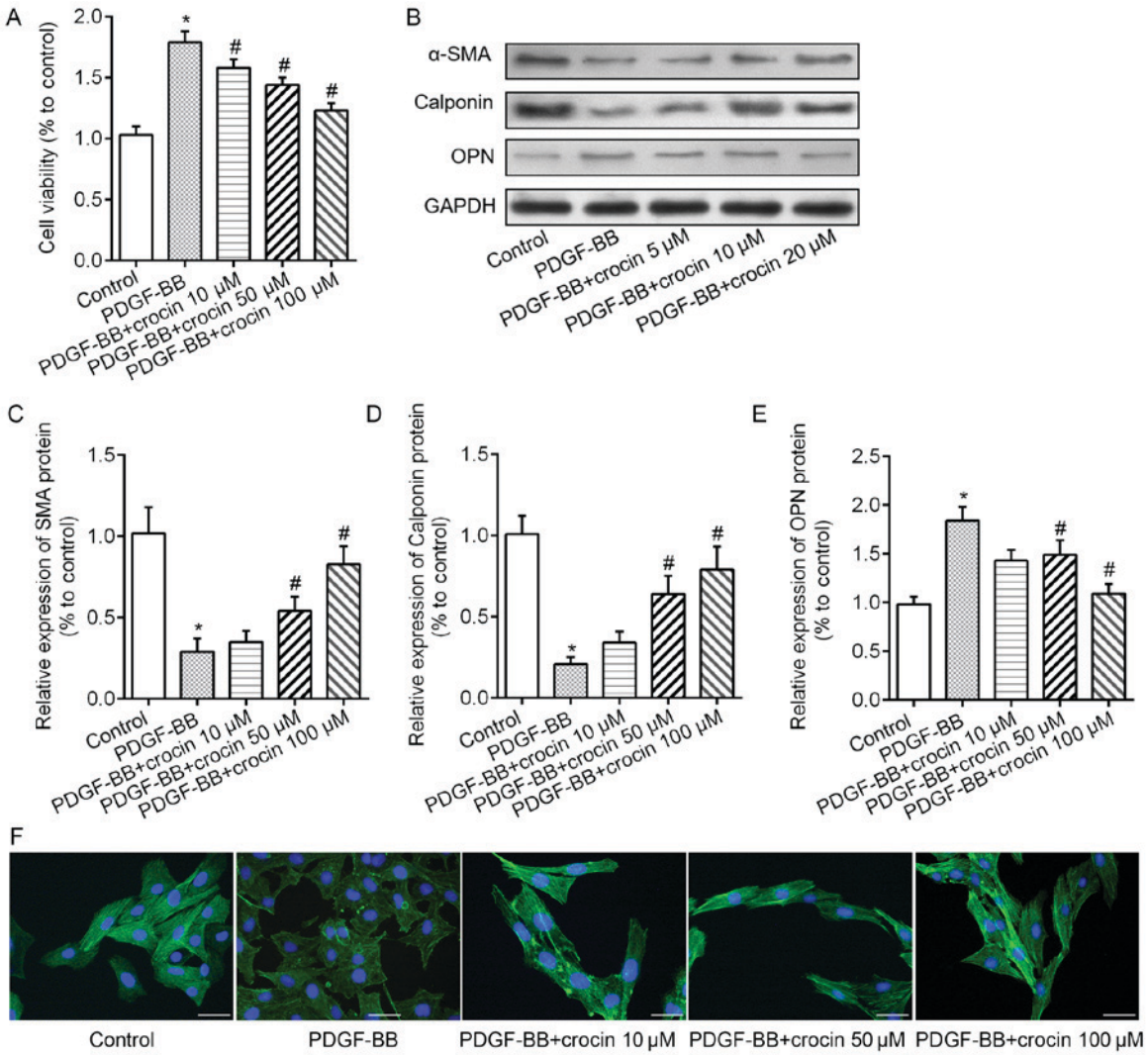

Figure 2. Crocin abrogated PDGF-BB-induced VSMCs proliferation and phenotypic switch. (A) Cell Counting Kit-8 assay was used for the detection of VSMC proliferation rates in different groups. (B) Western blot analysis was conducted to quantify the expression levels of contractile protein α-SMA, calponin and synthetic proteins OPN. Bar graph showing the relative protein levels of (C) α-SMA, (D) calponin and (E) OPN. (F) Immunofluorescent staining of α-SMA (green) of VSMCs in different groups. DAPI staining (blue) indicated the nucleus. Scale bar, 50 μm. *P<0.05 vs. control; $^{\#}$P<0.05 vs. PDGF-BB. OPN, osteopontin; SMA, smooth muscle actin; VSMC, vascular smooth muscle cell; PDGF-BB, platelet-derived growth factor-BB.

Crocin inhibits PDGF-BB-induced activation of JAK/STAT3 and ERK/KLF4 signaling pathways in VSMCs. We then examined the underlying mechanism of the inhibitory effect of crocin to VSMCs and JAK/STAT3 and ERK/KLF4 signaling pathways were investigated. Fig. 3A demonstrated that VSMCs exposure to PDGF-BB resulted in significant increases in the phosphorylation levels of JAK1, JAK2 and STAT3. Treatment of crocin dramatically downregulated PDGF-BB-induced

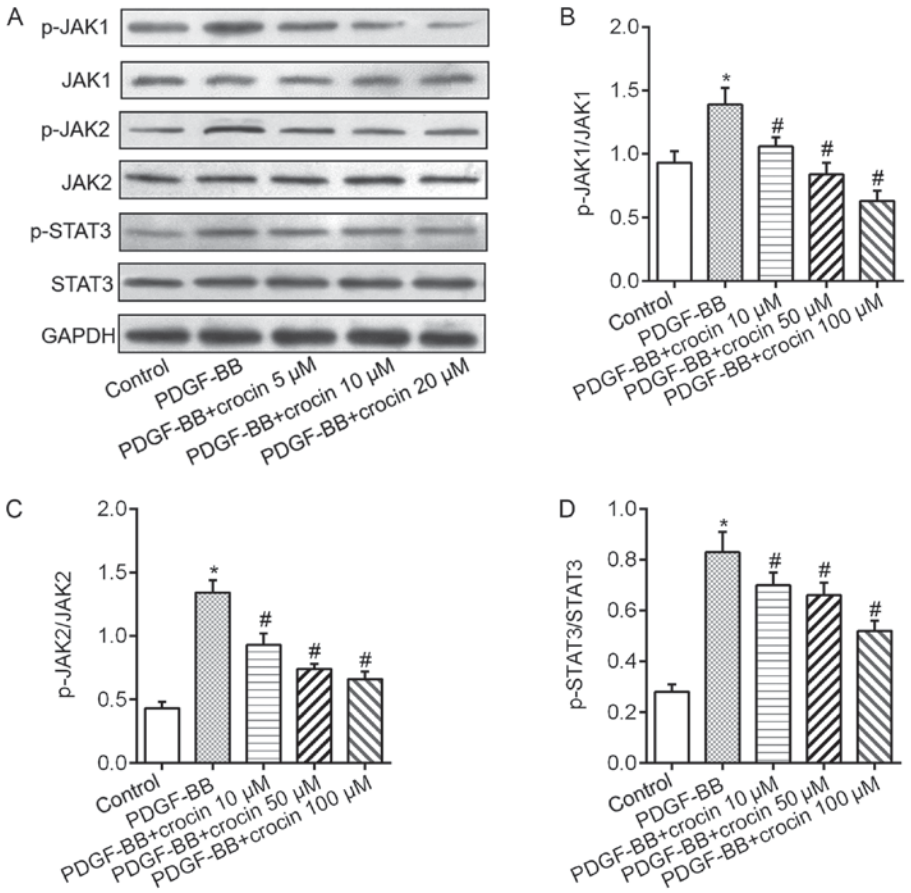

Figure 3. Crocin inhibited PDGF-BB-induced JAK/STAT3 signaling activation. (A) Representative images showing the protein expression of phosphorylated and total JAK1, JAK2 and STAT3. Bar graph showing the relative protein levels of phosphorylated (B) JAK1, (C) JAK2 and (D) STAT3. *P<0.05 vs. control; #P<0.05 vs. PDGF-BB. PDGF-BB, platelet-derived growth factor-BB; STAT3, signal transducers and activators of transcription; JAK, Janus kinase; p-, phosphorylated.

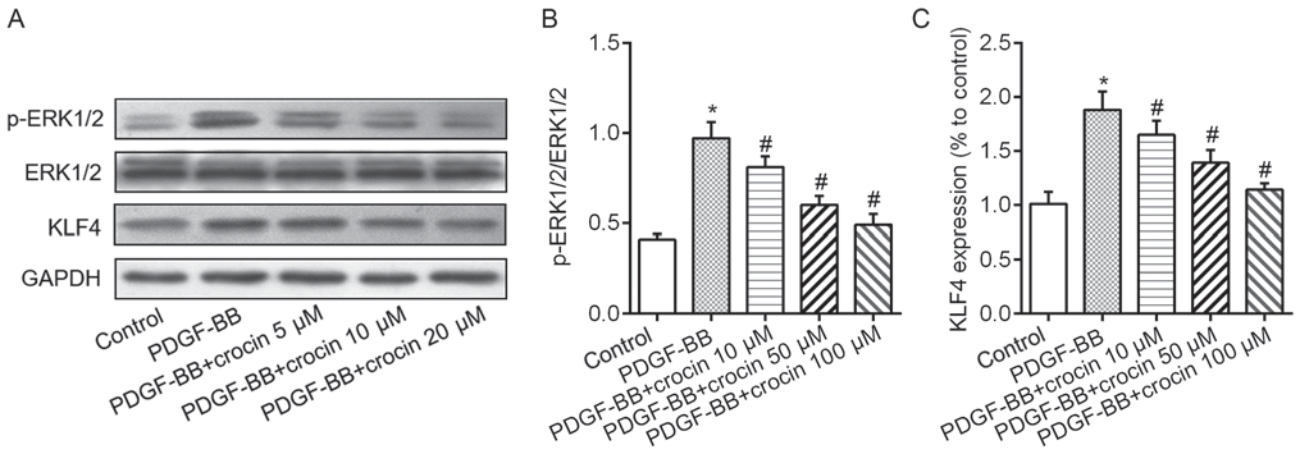

Figure 4. Crocin suppressed PDGF-BB-induced ERK/KLF4 signaling activation. (A) Representative images showing the protein expressions of phosphorylated and total ERK1/2 and expression levels of KLF4 protein. Bar graph showing the relative protein levels of (B) phosphorylated ERK1/2 and expression levels of (C) KLF4 in different groups. *P<0.05 vs. control; #P<0.05 vs. PDGF-BB. PDGF-BB, platelet-derived growth factor-BB; ERK, extracellular signal regulated kinase; KLF4, kruppel-like factor 4; p-, phosphorylated.

phosphorylation levels of JAK1, JAK2 and STAT3 in a concentration-dependent fashion (Fig. 3B-D). Additionally, in cultured VSMCs, exposure to PDGF-BB caused the elevated phosphorylation levels of ERK1/2 and increased expression levels of KLF4 protein (Fig. 4A). When the cells were co-treated with various concentration of crocin, above changes were abrogated in a concentration-dependent manner (Fig. 4B and C).

JAK/STAT3 and ERK/KLF4 signaling pathways mediate PDGF-BB-induced phenotype switching and VSMCs proliferation. We subsequently tested whether inhibition of the JAK/STAT3 and ERK/KLF4 signaling pathways were involved in PDGF-BB-induced phenotypic switching and proliferation of VSMCs. We found that administration of JAK inhibitor AG490 and ERK1/2 inhibitor U0126 significantly diminished

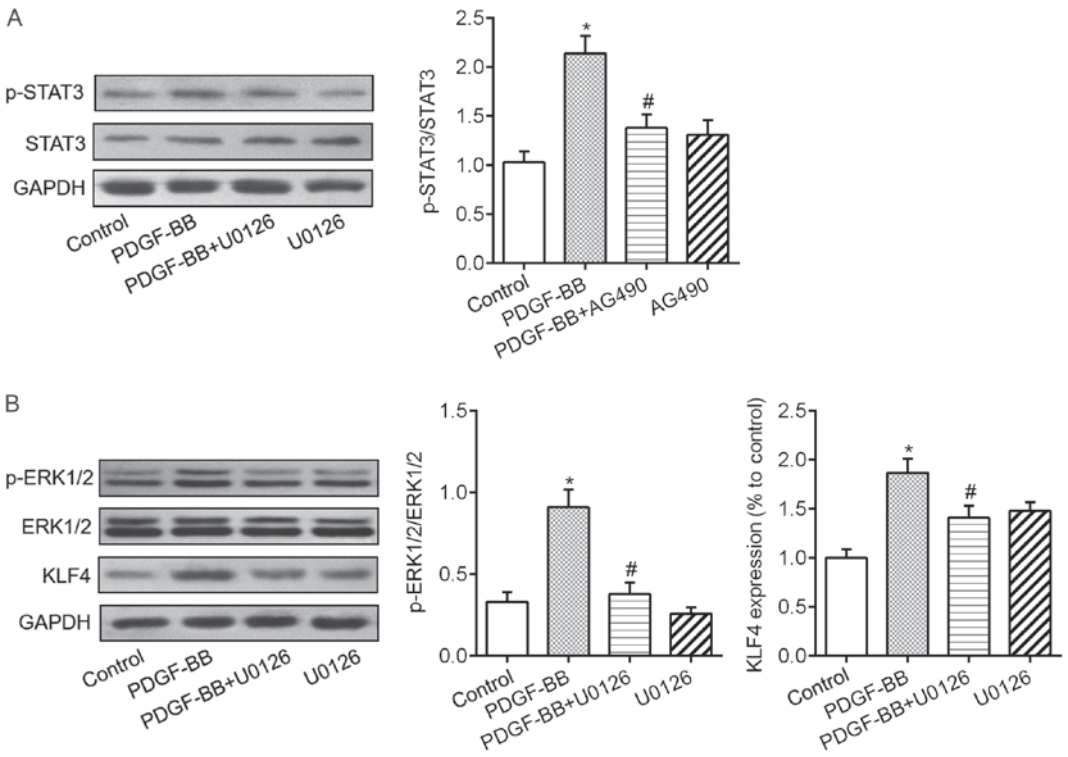

Figure 5. Suppression of PDGF-BB-induced JAK/STAT3 and ERK/KLF4 activation by AG490 and U0126 in VSMCs. (A) Representative western blot analysis showed phosphorylation of STAT3 in VSMCs treated with PDGF-BB (20 ng/ml) in the absence and presence of a specific JAK inhibitor AG490 (10 μM). (B) Representative images showed phosphorylation of ERK1/2 and expression levels of KLF4 protein in VSMCs treated with PDGF-BB (20 ng/ml) in the absence and presence of ERK1/2 inhibitor U0126 (50 μM). *P<0.05 vs. control; #P<0.05 vs. PDGF-BB. PDGF-BB, platelet-derived growth factor-BB; ERK, extracellular signal regulated kinase; KLF4, kruppel-like factor 4; p-, phosphorylated; STAT3, signal transducers and activators of transcription; JAK, Janus kinase; p-, phosphorylated; VSMC, vascular smooth muscle cell.

PDGF-BB-induced STAT3 phosphorylation (Fig. 5A), ERK1/2 phosphorylation and increased KLF4 levels (Fig. 5B). Meanwhile, the results of CCK-8 assay showed that blockade of JAK/STAT3 and ERK/KLF4 signaling pathways with AG490 or U0126 markedly attenuated PDGF-BB-induced VSMCs proliferation (Fig. 6A). Furthermore, the abnormal changes in phenotypic switching markers including α-SMA, calponin and OPN were all rectified by AG490 or U0126 pretreatment in VSMCs response to PDGF-BB (Fig. 6B-E). These findings suggested that both JAK/STAT3 and ERK/KLF4 signaling pathways were required for the role of crocin in VSMCs.

Discussion

The present findings revealed some novel findings about crocin. Crocin effectively suppressed PDGF-BB-induced VSMCs proliferation. In addition, crocin prevented PDGF-BB-induced the VSMCs phenotype switch through inhibiting JAK/STAT3 and ERK/KLF4 signaling pathway.

Researches demonstrated that PDGF-BB plays a vital role in the formation of neointima (15). In normal mature vessels, the expression of PDGF and its receptors are very low or undetectable. In contrast, its expression is dramatically increased at vascular injury sites, followed by the increased activation of platelets and the recruitment of monocytes (16). The expression of exogenous PDGF in the arteries can induce intimal thickening through the stimulation of VSMCs proliferation and migration (17). PDGF promoted VSMCs to switch from the quiescent contractile phenotype to the synthetic phenotype by inhibiting expression of α-SMA and calponin (18). Consistent with previous published studies, we found that PDGF-BB decreased α-SMA and calponin expression but increased OPN expression. The following experiments found that crocin restored the expression of α-SMA and calponin in a concentration dependent manner, accompanied by a decrease in cell proliferation. These data suggested that crocin halts the change toward a deleterious VSMCs phenotype induced by PDGF-BB. However, the mechanisms through which crocin inhibited PDGF-BB-induced VSMCs phenotypic switch remain unknown.

Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, which is one of major downstream mediators of PDGF signaling, was activated in VSMCs exposed to PDGF-BB in our experiments. Previous studies identified that STAT3 signaling pathway is required for PDGF-BB stimulated VSMC proliferation (19). And suppressing JAK/STAT3 signaling pathway leading to the inhibition of proliferation and migration of smooth muscle cells (20). We revealed that crocin inhibited the activation of JAK/STAT3 pathway induced by PDGF-BB in a concentration dependent manner. Actually, Kim *et al* have demonstrated that crocin effectively suppressed constitutive STAT3 activation, translocation of STAT3 to the nucleus through induction of protein tyrosine phosphatase SHP-1 in multiple myeloma cells (21). Accordingly, we identified that suppression of STAT3 activation using 100 μM crocin or a specific inhibitor resulted in VSMCs switching from the synthetic phenotype to the contractile phenotype again. The role of STAT3 in controlling

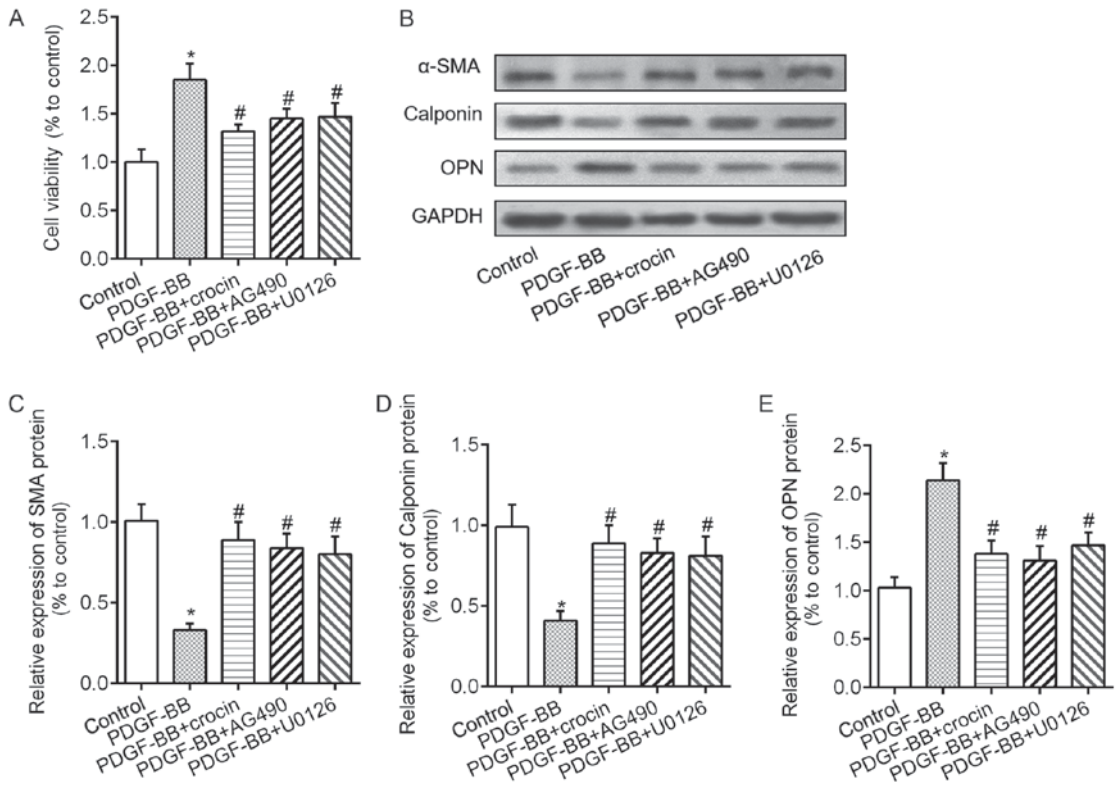

Figure 6. Crocin repressed PDGF-BB-induced VSMCs proliferation and phenotype switch via the JAK/signal transducers and activators of transcription 3 and ERK/kruppel-like factor 4 signaling pathways. (A) Cell Counting Kit-8 assay revealed cell proliferation in VSMCs treated with 20 ng/ml PDGF-BB, PDGF-BB + crocin (100 μM), PDGF-BB + AG490 (10 μM, a JAK inhibitor), PDGF-BB + U0126 (50 μM, an ERK inhibitor). (B) Representative images showed the expression levels of α-SMA, calponin and OPN protein. Bar graph showing the relative protein levels of (C) α-SMA, (D) calponin and (E) OPN in the groups. *P<0.05 vs. control; #P<0.05 vs. PDGF-BB. VSMC, vascular smooth muscle cell; PDGF-BB, platelet-derived growth factor-BB; ERK, extracellular signal regulated kinase; JAK, Janus kinase.

VSMCs phenotypic switch was supported by Liao *et al* study that activated STAT3 enhanced VSMCs proliferation and suppressed the expression of contractile proteins, whereas knockdown of endogenous STAT3 enhances VSMCs contractile phenotype (22). Although should be further validated *in vivo*, above data indicated that that JAK/STAT3 pathway was a potential therapeutic target for controlling phenotypic switch of VSMCs.

Besides activation of STAT transcription factors, PDGF-BB stimulation also leads to the initiation of the ERK signaling pathway (23,24). Studies by several investigators showed that activation of ERK1/2 signaling contributes to promote VSMC proliferation (25,26). KLF4 is the downstream target of ERK1/2 in the PDGF-BB-induced signaling pathway (27,28). Consistent with another study (29), we noted that PDGF-BB induces phosphorylation of ERK1/2 and elevated KLF4 expression in VSMCs. Moreover, crocin treatment significantly inhibited PDGF-BB-induced activation of ERK/KLF4 signaling pathway. It is noteworthy that crocin can inhibit p-ERK1/2 in rat retina with ischemia/reperfusion injury (30) and improve lipid dysregulation in subacute diazinon exposure through inhibition of ERK1/2 activation in rat liver (31). We for the first time showed that crocin suppressed ERK1/2 pathway in cultured VSMCs. The role of ERK1/2 and KLF4 in VSMCs phenotypic switch had been well established (32-35). We also revealed that ERK1/2 inhibitor U0126 significantly reduced p-ERK1/2 and KLF4 levels. Consistent with crocin, U0126 reversed PDGF-BB-induced VSMCs phenotypic switch. Nevertheless, whether there excites another pathway that control KLF4 expression and the exact upstream kinases of ERK1/2 is still need to be identified. Collectively, our results suggested that inhibiting PDGF-BB-induced activation of ERK/KLF4 signaling pathway may have contributed to the inhibition of VSMC proliferation and phenotypic switch exerted by crocin. Of note, studies found JAK/ERK/STAT pathway was associated with cell proliferation, differentiation and survival (36,37). The fact that JAK/ERK/STAT signaling pathway is also involved in attenuating cardiac ischemia/reperfusion injury (38) suggested an interaction between JAK and ERK signaling. Since PDGF-BB treatment influences other MAPK other MAPK pathways, such as JNK and p38 MAPK pathway (39), whether they participate in the inhibitory effects of crocin still needs to be defined.

Taken together, our results disclose for the first time that crocin inhibits PDGF-BB-induced VSMC phenotypic alteration and subsequent proliferation through regulating JAK/STAT3 and ERK/KLF4 signaling pathway. As VMSC proliferation is one of the key mechanisms involved in the development and progression of neointimal hyperplasia, which contributes to the pathogenesis of atherosclerosis and restenosis, use of crocin may be a potential way to restrain the progression of cardiovascular disease.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

GQ designed the study and performed the statistical analysis; LT conducted all experiments and data correction; GQ and LT wrote the manuscript.

Ethics approval and consent to participate

The study was approved by the Institutional Animal Care and Use Committee of the First Hospital of China Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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