Formononetin protects against balloon injury-induced neointima formation in rats by regulating proliferation and migration of vascular smooth muscle cells via the TGF-β1/Smad3 signaling pathway

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Abstract. The present study investigated the effects of formononetin (FMN) against balloon injury-induced neointima formation *in vivo* and platelet-derived growth factor (PDGF)-BB-induced proliferation and migration of vascular smooth muscle cells (VSMCs) *in vitro*, and explored the underlying mechanisms. A rat model of carotid artery injury was established, in order to examine the effects of FMN on balloon injury-induced neointima formation. Histological observation of the carotid artery tissues was conducted by hematoxylin and eosin staining. VSMC proliferation during neointima formation was observed by proliferating cell nuclear antigen staining. Subsequently, rat aortic VSMCs were isolated, and the effects of FMN on PDGF-BB-induced VSMC proliferation and migration were determined using Cell Counting Kit-8 and Transwell/wound healing assays, respectively. Immunohistochemical and immunocytochemical staining was applied to measure the expression of transforming growth factor (TGF)-β in carotid artery tissues and VSMCs, respectively. SMAD family member 3 (Smad3)/phosphorylated (p)-Smad3 expression was examined by western blotting. FMN treatment significantly inhibited the abnormal proliferation of smooth muscle cells in neointima, and alterations to the vascular structure were attenuated. In addition, pretreatment with FMN effectively inhibited the proliferation of PDGF-BB-stimulated VSMCs ($P<0.05$). FMN also reduced the number of cells that migrated to the lower surface of the Transwell chamber and decreased wound-healing percentage ($P<0.05$). The expression levels of TGF-β were decreased by FMN treatment *in vivo* and *in vitro*, and Smad3/p-Smad3 expression was also markedly inhibited. In conclusion, FMN significantly protected against balloon injury-induced neointima formation in the carotid artery of a rat model; this effect may be associated with the regulation of VSMC proliferation and migration through altered TGF-β1/Smad3 signaling.

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Introduction

Percutaneous transluminal coronary angioplasty (PTCA) is a commonly used clinical treatment for cardiovascular diseases; however, the frequent complication of restenosis following PTCA often limits the long-term beneficial effects of PTCA (1-3). The three relatively independent steps involved in the development of post-PTCA restenosis are as follows: Thrombosis, intimal hyperplasia, and vascular remodeling caused by post-injury vascular repair (3-5). Various vascular diseases, including neointimal hyperplasia following vascular intervention, are associated with abnormal vascular smooth muscle cell (VSMC) proliferation and migration (5,6-8). Previous studies have also reported that differentiation and activation of VSMCs and dysregulation of the extracellular matrix (ECM) may have critical roles in intimal hyperplasia, thus contributing to restenosis following balloon injury (9-11). Therefore, it may be beneficial to identify an effective treatment strategy against neointima formation and VSMC dysfunction following vascular endothelial injury.

Formononetin (FMN) is a bioactive compound, which is widely distributed in nature and has been reported to possess numerous pharmacological activities, including antitumor, oxygen free radical-scavenging, cell lipid peroxide-reducing and cholesterol-reducing effects (12-15). In our preliminary experiments, it was demonstrated that, in a rat model, FMN could attenuate balloon-induced neointima formation and reduce platelet-derived growth factor (PDGF) levels

(Song *et al*, unpublished data). PDGF is a potent growth factor associated with vascular development, which is produced by platelets, SMCs and endothelial cells in the injured vessel wall. At present, there are four known isoforms of PDGF: PDGF-A, PDGF-B, PDGF-C and PDGF-D, among which PDGF-A and PDGF-B can form homo- and heterodimers (16). The homodimer PDGF-BB is now considered to be the strongest factor in stimulating the proliferation of SMCs, and it can also induce production of ECM proteins by pericytes, which are important for the basement membrane of capillaries (17). It has also been reported that PDGF-BB is closely associated with cell migration in angiogenesis by regulating vascular endothelial growth factor expression levels in mural cells and collagenases in fibroblasts (18).

The present study aimed to investigate the effects of FMN against balloon injury-induced neointima formation *in vivo*, to determine its inhibitory effects on the proliferation and migration of VSMCs induced by PDGF-BB *in vitro*, and to assess the underlying mechanisms of these effects.

Materials and methods

Chemicals. FMN was obtained from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China); the purity of the chemical was 98%.

Animal grouping and treatment. Specific pathogen-free (SPF) grade healthy male Sprague-Dawley rats (age, 2.5-3.5 months; weight, 250-350 g) were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). Rats were maintained in a SPF grade, quiet and well-ventilated animal room under the following conditions: Temperature, 22-24˚C; relative humidity, 50-60%; 12-h light/dark cycle, for at least one week prior to the experiments. Rats were allowed *ad libitum* access to food and water. The present study was approved by the animal care committee of Linyi Peoples' Hospital Affiliated to Shandong University (Linyi, China), and all experimental operations were conducted according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (19).

A total of 30 rats were randomly divided into the sham group (n=10) and model group (n=20). Rats in the sham group were treated with intragastric administration of saline (10 ml/kg). Rats in the experimental group with balloon-induced arterial injury were divided into two groups (n=10/group): Model group (intragastric administration of 10 ml/kg saline) and FMN group (intragastric administration of 50 mg/kg FMN). Drug treatment was initiated on the second day after modeling, and drugs were administered once a day for 14 days.

Carotid artery injury model. A rat model of balloon-induced carotid artery injury was generated as described previously (20). Briefly, Sprague-Dawley rats were anesthetized with 10% chloral hydrate (300 mg/kg), fixed on an operating table in the supine position, and the left common and external carotid arteries were exposed and isolated. A 2 F balloon catheter was introduced retrogradely into the common carotid artery through the external carotid artery. The balloon was filled with saline and pulled back and forth; this process was repeated three times, in order to induce damage to the arterial endothelium. After surgery, the balloon catheter was removed and then the dorsal end of the external carotid artery was sutured to restore blood flow. Rats in the sham group were subjected to a similar surgical treatment; the left common and external carotid arteries were exposed, but the rats did not undergo balloon injury.

A total of 2 weeks following surgery, rats were euthanized and arterial segments were harvested for histological staining, identification of intimal hyperplasia and western blotting.

Histological observation. The carotid arteries were fixed with 10% neutral formaldehyde solution for 48 h at 4˚C (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and were embedded in paraffin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Carotid artery sections (5 μm) from each group were stained with hematoxylin and eosin. Briefly, after dewaxing with xylene and rehydration through a graded series of ethanol (Sigma-Aldrich; Merck KGaA), hematoxylin (Sigma-Aldrich; Merck KGaA) staining was performed for 5 min at 25˚C. After washing with tap water, tissue sections were treated with ethanol containing hydrochloric acid (Sigma-Aldrich; Merck KGaA). After immersion in tap water for 15 min, eosin (Sigma-Aldrich; Merck KGaA) staining was performed for 2 min. Subsequently, the slides were incubated in 95% anhydrous ethanol for 5 min twice and in xylene solution for 10 min at 25˚C, after which the slides were mounted in neutral resin and observed under an optical microscopy (BX51; Olympus Corporation, Tokyo, Japan). Morphometric analysis was performed by an investigator who was kept blind to the experimental procedure using three individual sections of arterial segments from each group. The Image-Pro Plus System 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) was used to measure the intima and media area of each group, and their ratio. Intima area = inner elastic membrane surrounding area - lumen area; media area = outer elastic membrane surrounding area - inner elastic membrane surrounding area.

Proliferating cell nuclear antigen (PCNA) staining. Carotid artery sections (5 μm) from each group were stained with PCNA, according to the manufacturer's protocol. Sections were incubated with the following antibodies: Anti-PCNA antibody (1:200, sc-53407; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4˚C overnight, after which biotinylated goat anti-mouse secondary antibody (1:200, sc-2039; Santa Cruz Biotechnology, Inc.) was added for 10 min at 37˚C. Each section was observed under an optical microscopy (BX51; Olympus Corporation), and six fields of view were analyzed. The number of PCNA-positive cells in the media and membranes of each vessel section were counted using Image-Pro Plus System 6.0 (Media Cybernetics, Inc.), the percentage was calculated, and the average value was determined as the number of PCNA-positive cells.

Cells and cell culture. Aortic VSMCs were isolated from healthy control rats, cultured and maintained as previously described (21). Cells were maintained and cultured in Dulbecco's modified Eagle's medium (DMEM, 5 mM glucose) supplemented with 10% fetal bovine serum (FBS) (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and

1% penicillin-streptomycin at 37˚C in a humidified environment containing 95% air and 5% CO_2. Cells used in subsequent experiments were obtained from passages 8 and 10.

MTT assay. Cells seeded in 96-well plates ($3x10^3$/well) were divided into the following six groups: Control group (without FMN or PDGF-BB treatment), FMN group (treated with 20 μM FMN for 48 h at 37˚C), PDGF-BB group (treated with 40 ng/ml PDGF-BB for 48 h at 37˚C), and FMN + PDGF-BB groups (pretreated with 5,10 or 20 μM FMN for 24 h, followed by treatment with PDGF-BB at a final concentration of 40 ng/ml for 24 h at 37˚C). Subsequently, an MTT assay was performed by adding 20 μl MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA) to the cells for 4 h at 37˚C. The supernatant was then discarded, 150 μl dimethyl sulfoxide was added to each well, the plates were agitated for 10 min and light absorbance was measured at 570 nm using a microplate reader (PerkinElmer, Inc., Waltham, MA, USA).

Wound-healing assay. VSMCs were seeded into 6-well plates ($3.0x10^5$ cells/well) and were treated as aforementioned (cells in the FMN + PDGF-BB group were pretreated with 20 μM FMN for 24 h, and were then treated with PDGF-BB at a final concentration of 40 ng/ml for 24 h). Once the cells adhered to the dishes and just before PDGF-BB was added, a sterile plastic 10-μl micropipette tip was used to generate one homogeneous wound through the cell layer, and the cells were washed twice with PBS. The wound width in each group was measured under a microscope (IX51; Olympus Corporation) and images were captured at 100x magnification; images of the initial wound width and the wound width after 24 h were obtained. Wound healing percentage (%) = (initial wound width - 24 h wound width)/initial wound width x 100.

Cell migration assay. VSMCs were seeded into the upper well of a Transwell plate (10^5/well) in 200 μl serum-free DMEM, and the bottom chambers were filled with 600 μl DMEM containing 10% FBS with saline, FMN and/or PDGF-BB as aforementioned. Cells were incubated for 24 h at 37˚C after all the treatments were implemented. Subsequently, the chambers were washed gently with PBS, cells were gently removed from the upper surface of the chamber with a cotton swab, and the remaining cells in the lower surface of the chamber were fixed in methanol for 20 min and stained with crystal violet. Cells that had migrated and remained on the lower surface of the chamber were counted under a microscope (BX51; magnification, x100; Olympus Corporation).

Immunohistochemistry. Carotid artery sections (5 μm) from each group were dewaxed and dehydrated. Subsequently, the sections were incubated with 3% hydrogen peroxide in methanol to block endogenous peroxidase and nonspecific binding was blocked with 5% bovine serum albumin at room temperature for 10 min (Gibco; Thermo Fisher Scientific, Inc.). The sections were then incubated with anti-transforming growth factor (TGF)-β1 primary antibody (1:200, ab92486; Abcam, Cambridge, UK) at 4˚C overnight. An SABC kit (SA1025) and a DAB kit (AR1022) (both from Wuhan Boster Biological Technology, Ltd., Wuhan, China) were used for subsequent reactions, according to the manufacturer's protocols. Five sections of each tissue were observed under an optical microscope (magnification, x400; Olympus Corporation), and Image-Pro Plus System 6.0 (Media Cybernetics, Inc.) was used to analyze positive cell counts.

Immunocytochemistry. VSMCs were seeded on cover slips in the presence of DMEM containing 10% FBS in 6-well plates, and were treated as aforementioned. The expression levels of TGF-β1 in the cells were detected using an anti-TGF-β1 primary antibody (1:100, ab92486; Abcam) at 4˚C overnight. Subsequently, cells were incubated with a biotinylated goat anti-rabbit secondary antibody (1:200, ab97049; Abcam) at 37˚C for 10 min. DAB kit (AR1000; Wuhan Boster Biological Technology, Ltd.) was used for subsequent reactions, according to the manufacturer's protocols. The results were analyzed in the same manner as immunohistochemistry staining.

Western blotting. Western blotting was applied to measure the expression levels of SMAD family member 3 (Smad3) and phosphorylated (p)-Smad3 in carotid arteries from rats in each group, and in VSMCs exposed to various treatments *in vitro*. For the *in vitro* and *in vivo* assays, VSMCs and carotid artery tissues were harvested and lysed with Radioimmunoprecipitation Assay Lysis Buffer (Beyotime Institute of Biotechnology, Haimen, China). The protein samples were quantified using a bicinchoninic acid protein assay kits (Beyotime Institute of Biotechnology) and were separated by 10% SDS-PAGE (30 μg protein/lane). Following electrophoresis, proteins were electrotransferred onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). The PVDF membranes were blocked with 5% skim milk powder solution for 1.5 h at room temperature. Protein levels were detected using the following primary antibodies: Rabbit anti-Smad3 (1:500, ab40854; Abcam), rabbit anti-p-Smad3 (1:200, ab52903; Abcam) and mouse anti-β-actin (1:5,000, ab6276; Abcam), which was used as an internal control. Following incubation with primary antibodies at room temperature for 2 h, PVDF membranes were washed in 0.05% Tween-20/Tris-buffered saline, and incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit: 1:2,000, ZB-2301; goat anti-mouse: 1:5,000, ZDR-5307; OriGene Technologies, Inc., Beijing, China) at room temperature for 1 h. The bound antibodies were visualized using an enhanced chemiluminescence reagent (EMD Millipore). Relative protein expression levels were semi-quantified by densitometric analysis using the ChemiDoc XRS+ image analyzer (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. All data from at least three independent experiments were expressed as the means ± standard deviation. SPSS 19.0 statistical software (IBM Corp., Armonk, NY, USA) was used to analyze the variance in data. Data were analyzed using one-way analysis of variance followed by the least significant difference post-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

FMN prevents neointima formation in a balloon-induced carotid artery injury model. Typical histological analysis

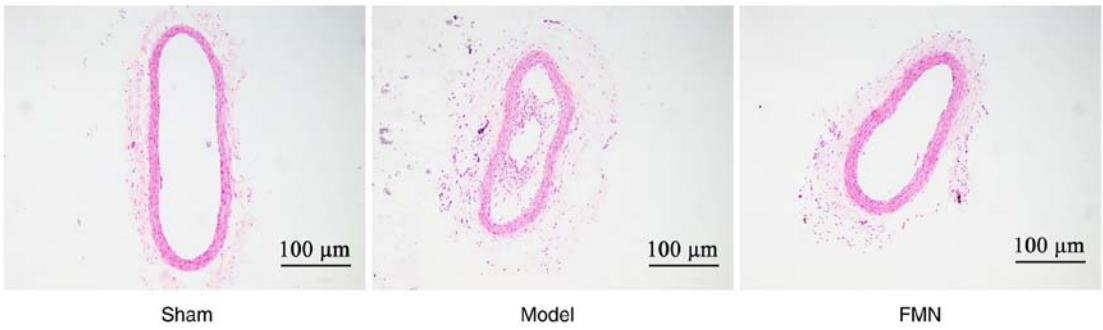

Figure 1. Histological analysis using hematoxylin and eosin staining of carotid artery sections from each group (magnification, x100; scale bar, 100 μm). FMN, formononetin.

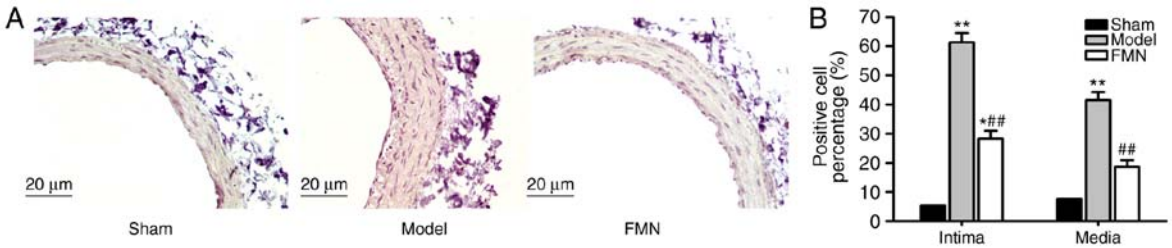

Figure 2. PCNA staining of carotid artery sections from each group. (A) PCNA staining (magnification, x400; scale bar, 20 μm). (B) PCNA-positive cell percentage in the intima and media. **P<0.01 vs. the sham group; *P<0.05 vs. the sham group; $^{\#\#}$P<0.01 vs. the model group. FMN, formononetin; PCNA, proliferating cell nuclear antigen.

Table I. Quantitative analysis of neointima formation.

Group	Intima area (mm^2)	Media area (mm^2)	Intima/media area ratio
Model	19.2±3.4	9.8±1.6	1.96±0.27
Formononetin	3.3±0.4[a]	7.2±1.1	0.45±0.11[a]

[a]P<0.05 vs. the model group.

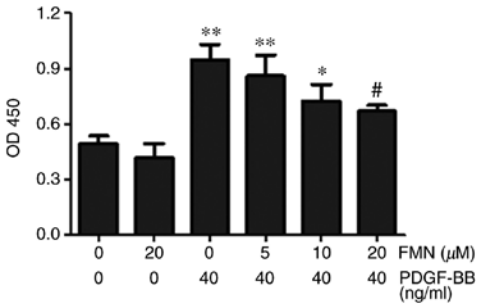

Figure 3. FMN inhibits PDGF-BB-induced vascular smooth cell proliferation. **P<0.01 vs. the control group; *P<0.05 vs. the control group; $^{\#}$P<0.05 vs. the PDGF-BB group. FMN, formononetin; OD, optical density; PDGF-BB, platelet derived growth factor-BB.

using hematoxylin and eosin staining detected marked structural alterations to the vasculature and narrowing of the vessel cavity in the model group compared with in the sham group. As shown in Fig. 1, in the model group, serious intimal hyperplasia was observed, alongside thickened intima and matrix accumulation, incomplete intimal repair, discontinuous internal elastic lamina and roughened inner surface of blood vessels. In addition, VSMCs exhibited abnormal proliferation and migration from media to the intima, thus indicating successful establishment of the carotid artery injury model. Conversely, in the FMN treatment group, abnormal proliferation of VSMCs in neointima was markedly reduced, and alterations to the vascular structure were attenuated. Quantitative analysis of neointima formation demonstrated that, in the FMN treatment group, intima area and intima/media area ratio were decreased compared with in the model group (Table I). These findings suggested that FMN may significantly prevent abnormal neointimal hyperplasia in a balloon-induced carotid artery injury model.

VSMC proliferation in the intima of a balloon-induced carotid artery injury model. The results of carotid artery PCNA staining are presented in Fig. 2; positive cell nuclei were stained brown. Large amounts of cell proliferation were detected in the intima and media of the model group (Fig. 2A). The positive cell rate was significantly increased compared with in the sham group (P<0.01). After FMN treatment, the number of PCNA-positive cells was significantly reduced compared with in the model group (Fig. 2B). These results indicated that FMN treatment may inhibit abnormal intimal hyperplasia.

FMN inhibits PDGF-BB-induced VSMC proliferation. The present results demonstrated that PDGF-BB treatment alone could stimulate the proliferation of VSMCs, as revealed by a significantly increased optical density value compared with in the control group. Conversely, pretreatment with FMN

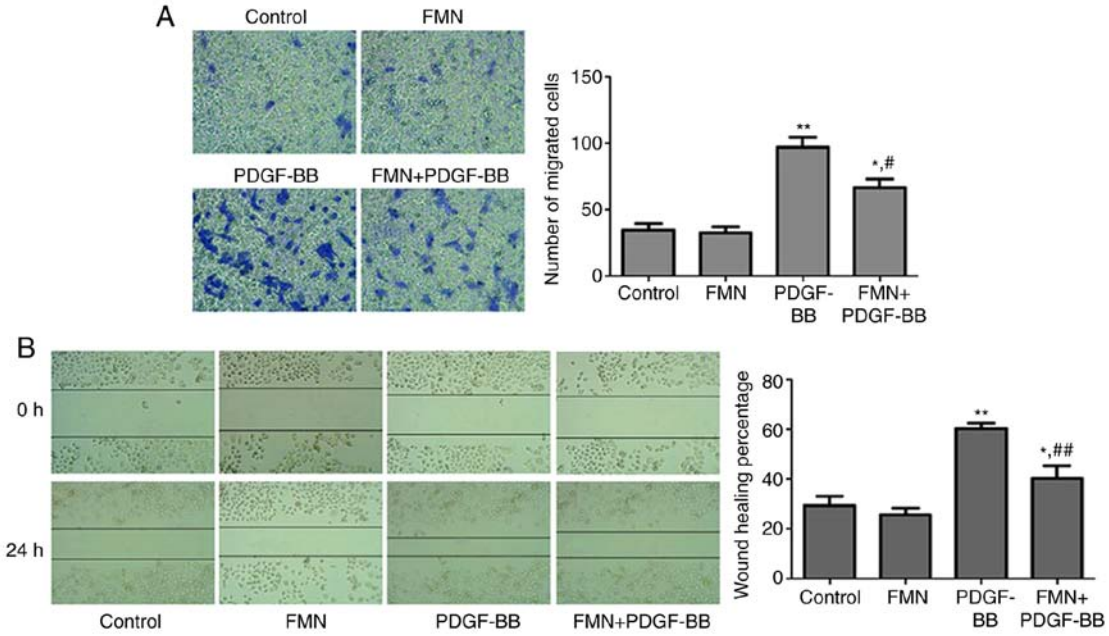

Figure 4. Effects of FMN on vascular smooth cell migration. (A) Transwell migration assay (magnification, x100). (B) Wound-healing assay (magnification, x100). Data are presented as the means ± standard deviation of triplicate experiments (n=3). **P<0.01 vs. the control group; *P<0.05 vs. the control group; $^{\#\#}$P<0.01 vs. the PDGF-BB group; $^{\#}$P<0.05 vs. the PDGF-BB group. FMN, formononetin; PDGF-BB, platelet derived growth factor-BB.

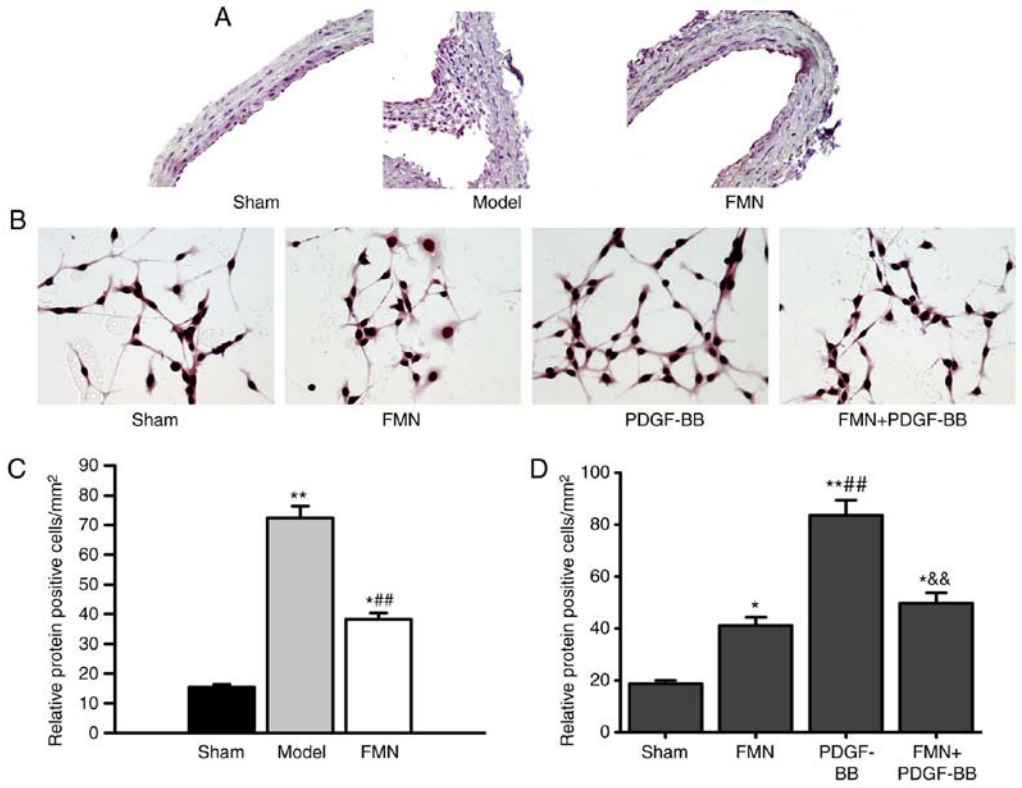

Figure 5. Regulatory effects of FMN on TGF-β1 expression *in vivo* and *in vitro*. (A) FMN decreased TGF-β1 expression in carotid artery tissues following balloon-injury (magnification, x400). (B) FMN decreased TGF-β1 expression induced by PDGF-BB in VSMCs (magnification, x400). (C) Semi-quantitative analysis of TGF-β1 expression in carotid artery tissues. **P<0.01 vs. the Sham group; *P<0.05 vs. the Sham group; $^{\#\#}$P<0.01 vs. the Model group. (D) Semi-quantitative analysis of TGF-β1 expression in VSMCs. **P<0.01 vs. the Sham group; *P<0.05 vs. the Sham group; $^{\#\#}$P<0.05 vs. the FMN group; $^{\&\&}$P<0.01 vs. the PDGF-BB group. FMN, formononetin; PDGF-BB, platelet derived growth factor-BB; TGF-β1, transforming growth factor- β1; VSMCs, vascular smooth muscle cells.

effectively inhibited PDGF-BB-stimulated proliferation of VSMCs, and the maximum inhibitory effect was observed in the 20 μM FMN pretreatment group; the findings were not significantly different compared with in the control group. In addition, no notable cytotoxicity of FMN was detected, and treatment with 20 μM FMN alone did not significantly affect

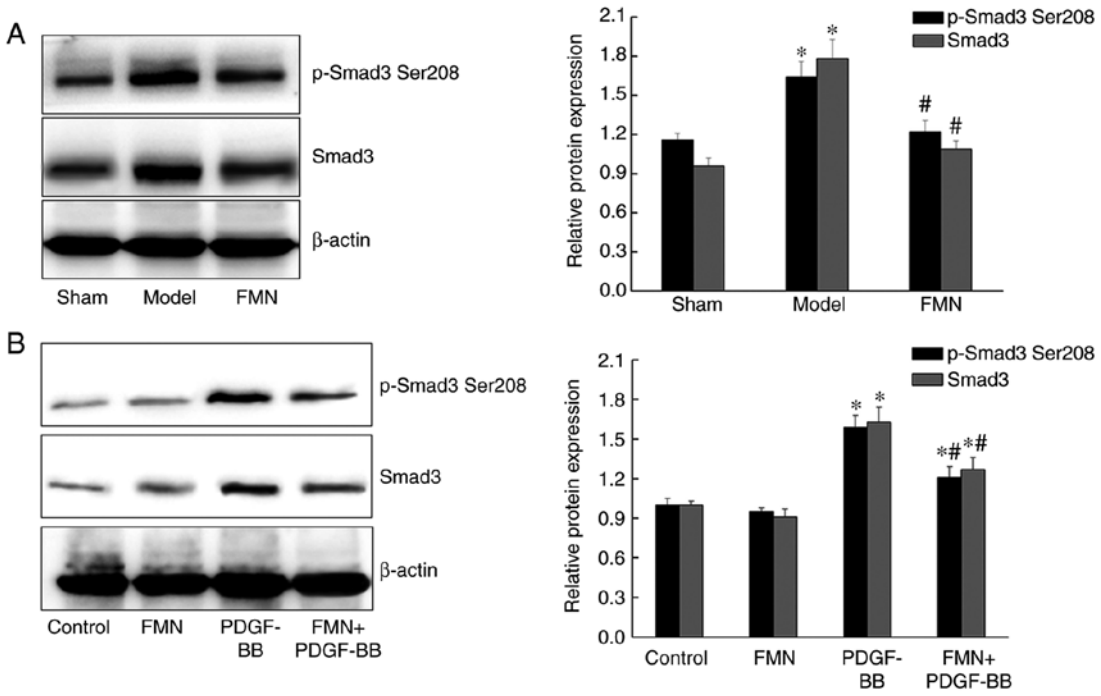

Figure 6. FMN regulates Smad3 expression *in vivo* and *in vitro*. (A) FMN downregulated Smad3/p-Smad3 expression in carotid artery tissue from rats with balloon-induced carotid artery injury. *P<0.05 vs. the sham group; #P<0.05 vs. the model group. (B) FMN decreased elevated Smad3/p-Smad3 expression induced by PDGF-BB stimulation in vascular smooth muscle cells. *P<0.05 vs. the control group; #P<0.05 vs. the PDGF-BB group. FMN, formononetin; PDGF-BB, platelet derived growth factor-BB; p-Smad3, phosphorylated-Smad3; Smad3, SMAD family member 3.

the cell proliferation of VSMCs compared with in the control group (P>0.05; Fig. 3).

FMN regulates PDGF-BB-induced VSMC migration. The inhibitory effects of FMN on SMC migration were examined using a Transwell plate with filters (pore size, 8 μm). The results demonstrated that PDGF-BB could significantly induce SMC migration (Fig. 4), which was attenuated by treatment with FMN. The number of migrated cells in the FMN treatment group was slightly decreased; however, there was no statistically significant difference compared with in the control group (P>0.05). Subsequently, the migratory ability of VSMCs was confirmed using a wound-healing assay; similar trends were observed as in the Transwell assay. These data suggested that FMN may significantly decrease the migratory ability of VSMCs induced by PDGF-BB.

TGF-β1 expression is decreased by FMN treatment in vivo and in vitro. The expression levels of TGF-β1 in carotid artery tissues and PDGF-BB-treated cells were detected by immunohistochemistry and immunocytochemistry, respectively (Fig. 5). As shown in Fig. 5A and C, the expression levels of TGF-β1 in the carotid artery tissues of the model group were markedly elevated compared with in the sham group. FMN treatment significantly attenuated the increased expression of TGF-β1 in the carotid artery tissues. To confirm whether FMN could modulate TGF-β1 expression following artery injury, PDGF-BB stimulation in VSMCs was used to imitate injury *in vitro*. The data indicated that treatment with PDGF-BB markedly increased the expression levels of TGF-β1 in VSMCs, whereas FMN was able to neutralize the effects of PDGF-BB (Fig. 5B and D).

FMN regulates Smad3 expression in vivo and in vitro. To further investigate whether FMN may modulate TGF-β1/Smad3 signaling, Smad3 and p-Smad3 expression was detected in carotid artery tissues and VSMCs. As shown in Fig. 6A, in rats with balloon-induced carotid artery injury, Smad3 and p-Smad3 expression levels were elevated, whereas FMN treatment significantly reduced the expression levels. In addition, increased Smad3 and p-Smad3 expression was detected in VSMCs treated with PDGF-BB, whereas FMN was able to block the stimulatory effects of PDGF-BB. In the FMN treatment group, Smad3 and p-Smad3 expression was slightly reduced compared with in the control group; however, this was not statistically significant (P>0.05).

Discussion

Restenosis is a major limitation of the application of arterial reconstruction procedures, including balloon angioplasty, stenting and coronary artery bypass; during the process of restenosis formation, SMCs migrate from the media toward the intima, where they abnormally proliferate and undergo phenotypic changes (22,23). The main findings of the present study were as follows: i) Marked neointima formation was detected in a rat model of balloon-induced carotid artery injury, and FMN treatment significantly prevented neointima formation; ii) *in vitro* experiments confirmed that FMN markedly inhibited the abnormal proliferation of PDGF-BB-induced VSMCs; iii) the mechanisms underlying the effects of FMN on the inhibition of neointima formation in a rat model of balloon-induced carotid artery injury, and the proliferation of PDGF-BB-induced VSMCs, may be associated with regulation of the TGF-β/Smad3 signaling pathway.

FMN is a natural isoflavone compound that exists in numerous medicinal plants, including Caulis Spatholobi, *Astragalus membranaceus* and *Trifolium pretense* (24-26). Previous studies have revealed that FMM and its derivatives exhibit cardioprotective (27), vasorelaxation (28,29) and anti-hypertensive effects (29). Furthermore, it has been suggested that FMN, as a phytoestrogen, may inhibit vascular remodeling and neointima formation, thus protecting the cardiovascular system, which may be clinically useful in preventing cardiovascular disease in women and men as a safer substitute for feminizing estrogens (30). Based on the existing literature, it may be hypothesized that FMN possesses the ability to ameliorate post-PTCA restenosis.

In our preliminary experiments, it was observed that FMN could attenuate balloon-induced neointima formation, inhibit platelet aggregation and reduce PDGF levels in a rat model of balloon-induced carotid artery injury. In the present study, the inhibitory effects of FMN were confirmed on neointima formation in a rat model, as revealed by attenuated alterations in vascular structure in the injured carotid artery. In addition, FMN-induced inhibition of PDGF-BB-stimulated SMC proliferation and migration further verified the potential bioactivity of FMN. The effects of FMN on proliferation were slightly larger, as cell proliferation may be faster than migration.

To understand these effects, the present study further investigated the mechanisms underlying the activity of FMN. It has been well documented that PDGF and TGF-β1 are critical growth factors that have important roles in regulating SMC migration and proliferation under pathological conditions (31). TGF-β1, which is a highly complex polypeptide and an important member of the TGF-β superfamily, is believed to have multifunctional properties that serve critical roles in the pathophysiology of major diseases, including cardiovascular diseases (32,33). In a previous study, it was revealed that TGF-β1 expression is observed at an early stage of acute injury until day 14 after injury, and is decreased by 21 days (34). The present study detected elevated secretion of TGF-β1 in the rat model, which could be reversed by FMN treatment. Furthermore, in the VSMCs *in vitro* assay, a similar expression profile of TGF-β1 was detected after PDGF-BB stimulation as that detected *in vivo*.

Previous studies have reported that the TGF-β1/Smad3 signaling pathway is closely associated with neointima formation following vascular injury (34-36). It has been revealed that TGF-β1 may be involved in restenosis by recruiting mesenchymal stem cells following arterial injury (34), and stimulating fibronectin and collagen I/III synthesis in VSMCs via the Smad3 signaling pathway, thus resulting in ECM deposition in the neointima (35) and restenosis (36). It has also been reported that inhibition of Smad3 expression may compromise the proliferation of VSMCs (37). Furthermore, it has been reported that blocking TGF-β1/Smad3 signal transduction in a rat model of balloon-induced carotid artery injury could inhibit intimal hyperplasia (38). In the present study, it was revealed that FMN could alter the TGF-β1/Smad3 signaling pathway *in vivo* and *in vitro*. These results indicated that FMN may exert its effects via affecting the TGF-β1/Smad3 signaling pathway, which in turn may markedly inhibit VSMCs proliferation and migration.

In conclusion, in addition to its previously demonstrated pharmacological activities, the present study confirmed the FMN affects neointima formation in a rat model of balloon-induced carotid artery injury *in vivo* and PDGF-BB-induced VSMC proliferation and migration *in vitro*; this study provided pre-clinical data to suggest a novel clinical use for the natural isoflavone FMN. Furthermore, to the best of our knowledge, the present study was the first to demonstrate that FMN could adjust PDGF/TGF-β1/Smad3 signaling *in vitro* and *in vivo*, which may be a possible mechanism underlying the effects of FMN on neointima formation following intimal injury. The detailed mechanisms regarding how FMN regulates PDGF/TGF-β1/Smad3 signaling transduction require further in-depth investigation.

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Availability of data and materials

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

Authors' contributions

TS and YL participated in the design of the study and performed the statistical analysis. TS, YL, JZ, TJ, XJ and XL carried out the immunoassays and conceived the study, and participated in its design and coordination. TS, YL and XL drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by Linyi Peoples' Hospital affiliated to Shandong University animal care committee.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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