

ORIGINAL ARTICLE

Emodin prevents intima thickness via Wnt4/Dvl-1/ β -catenin signaling pathway mediated by miR-126 in balloon-injured carotid artery rats

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Neointimal proliferation after vascular injury is a key mechanism of restenosis, a major cause of percutaneous transluminal angioplasty failure and artery bypass occlusion. Emodin, an anthraquinone with multiple physiological activities, has been reported to inhibit proliferation of vascular smooth muscle cells (VSMCs) that might cause intimal arterial thickening. Thus, in this study, we established a rat model of balloon-injured carotid artery and investigated the therapeutic effect of emodin and its underlying mechanism. Intimal thickness was analyzed by hematoxylin and eosin staining. Expression of Wnt4, dvl-1, β -catenin and collagen was determined by immunohistochemistry and/or western blotting. The proliferation of VSMC was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and electron microscopy. MicroRNA levels were quantified by real-time quantitative PCR. Emodin relieved injury-induced artery intimal thickness. Results of western blots and immunohistochemistry showed that emodin suppressed expression of signaling molecules Wnt4/Dvl-1/ β -catenin as well as collagen protein in the injured artery. In addition, emodin enhanced expression of an artery injury-related microRNA, miR-126. *In vitro*, MTT assay showed that emodin suppressed angiotensin II (AngII)-induced proliferation of VSMCs. Emodin reversed AngII-induced activation of Wnt4/Dvl-1/ β -catenin signaling by increasing expression of miR-126 that was strongly supported by transfection of mimic or inhibitor for miR-126. Emodin prevents intimal thickening via Wnt4/Dvl-1/ β -catenin signaling pathway mediated by miR-126 in balloon-injured carotid artery of rats.

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INTRODUCTION

Stent implantation via arterial blood vessel has revolutionized the interventional therapy of obstructive artery disease. Although drug-eluting stents have dramatically reduced occurrence of restenosis,^{1–3} it is still a major limitation for the long-term prognosis after arterial surgery. It has been widely accepted that restenosis occurs because of proliferation of medial vascular smooth muscle cells (VSMCs) and migration and formation of large extracellular matrix secretion, resulting in neointimal thickness.⁴

Emodin (1,3,8-trihydroxy-6-methyl-anthraquinone), an anthraquinone with multiple physiological activities, has been widely reported to play roles in inhibiting cell proliferation, inducing apoptosis and suppressing migration, invasion and angiogenesis in cancers.^{5–8} Emodin has also been demonstrated to inhibit VSMC proliferation,^{9–11} implying a promising therapy of emodin for preventing restenosis. However, *in vivo* study of anti-restenosis with emodin is lacking, and the mechanism involved remains undefined.

The family of Wnt proteins, which were first identified in *Drosophila* wingless mutants, has a well-established role in embryogenesis and development. Emerging data show that Wnt proteins also regulate VSMC proliferation, migration and survival.^{12–14} As a dependent factor for canonical Wnt signaling, β -catenin expression and activation has been reported to be related with proliferation of VSMCs *in vitro* and *in vivo* after balloon injury of the rat carotid artery.^{12,15–17} However, the involvement of Wnt/ β -catenin signaling in emodin regulation of arterial restenosis remains to be explored.

Wnt/ β -catenin signaling has been reported to be involved with microRNA (miRNA) regulation of gene expression in cancer.¹⁸ The miRNAs are a class of highly conserved, single-stranded, noncoding small RNAs that control cellular function by either degrading mRNAs or inhibiting their translation. It has been reported that the miRNAs, as powerful regulators of gene expression, are involved in the modulation of VSMC migration and dedifferentiation and have crucial roles in intimal thickening after vascular injury.¹⁹ In response to

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vascular injury, miRNAs contribute to the formation of neointimal lesions and exhibit a dynamic profile in injured vessel walls.^{20,21} Little is known about the regulatory role of miRNAs on Wnt/ β -catenin signaling pathway in injured arteries.

In this study, we established a rat model for balloon-injured carotid artery and aimed to evaluate the role of emodin in intimal thickening *in vivo*. We directly assessed whether Wnt/ β -catenin signaling was involved in this process and aimed to identify the regulatory relationship between miRNAs and Wnt/ β -catenin signaling in the emodin-induced inhibition of VSMC proliferation.

MATERIALS AND METHODS

Reagents

Emodin was obtained from Shanghai Yuanye Bio-Technology Co., Ltd (purity >98%; batch lot, 20110323; Shanghai, China). Angiotensin II (AngII), a stimulant for proliferation for VSMCs, was obtained from Sigma (St Louis, MO, USA). LiCl, an inhibitor for Wnt/ β -catenin pathway, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. The primers for miRNAs were synthesized by Sangon (Shanghai, China). Antibodies against DVL-1, COL1A1(C-18), COL3A1(C-15) and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies to β -catenin and Wnt4 (rabbit monoclonal antibody) were purchased from Cell Signaling Technology (Danvers, MA, USA). All of the cell culture media and other reagents were from Invitrogen (Shanghai, China).

Animal models of intimal thickening

A total of 33 Sprague-Dawley rats (weight 350–400 g, purchased from the Animal Experiment Center of Zhejiang Chinese Medicine University, Hangzhou, China) were randomized into three groups. The first group was the operation group ($n=11$) in which animals were anesthetized by intraperitoneal injection of 30 mg kg⁻¹ pentobarbital, and an incision was made in the center of the neck. After the jugular vein was injected with 100 U kg⁻¹ heparin, the common carotid artery was isolated using blunt separation followed with temporary occlusion by vascular clamps at the proximal ends of the right common carotid arteries. A 1.5×12 mm PTCA balloon catheter (Medtronic, Minneapolis, MN, USA) was inserted gently from the incision on external carotid arteries ~2.0 cm into the common carotid arteries. The artery was injured by balloon inflation at 5 atm, followed by drawing back and pushing forward 5 times. After damage, the catheter and ligated external carotid arteries were withdrawn, the bulldog clamp (Medtronic) was removed to restore blood flow and the incision was sutured. The rats were resuscitated and then maintained with normal diet feeding. The second group was a sham operation group ($n=11$) in which the animals were operated according to above method without undergoing artery injury by balloon catheter. The third group was the emodin group ($n=11$) in which 40 mg kg⁻¹ day emodin (obtained from Yuanye Bio-Technology) was given by intragastric administration for 1 week before balloon injury and sustained for 6 weeks after operation. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 93-23, revised 1985) and were approved by the institutional care and use committee.

Histochemistry and immunohistochemistry

Ligated carotid arteries were removed at 2 or 6 weeks after balloon injury and embedded longitudinally in paraffin wax to assess intimal and medial lesion size by hematoxylin and eosin staining. Image analysis was performed with Image Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA), and the intimal/medial thickness was calculated as follows: intimal thickness = (internal plastic layer surrounding area/ π)^{1/2} - (luminal area/ π)^{1/2}, and medial thickness = (external plastic layer surrounding area/ π)^{1/2} - (internal plastic layer surrounding area/ π)^{1/2}. Each measurement was taken 3 times to obtain the mean.

Immunohistochemistry was performed on rat carotid artery paraffin wax-embedded sections using primary antibodies against Wnt4 (rabbit polyclonal antibody, Bioss Inc., Woburn, MA, USA). Nonimmune IgG was used as a negative control. Biotin-labeled goat-anti-rabbit IgG served as the secondary antibody. Wnt4 was located on the cellular membrane as well as in the cytoplasm.

VSMC culture

Aortas were obtained from Sprague-Dawley rats, and VSMCs were grown from aortic explants and used at passages 3 to 6. Cells were treated with different conditions when they reached 75–85% confluence: (1) AngII (Sigma) at 1×10⁻⁶ mol l⁻¹ (determined by prestudy) for 48 h; (2) emodin (Pfizer, Groton, CT, USA) 10, 40 and 80 μ mol l⁻¹ for 48 h; (3) miR-126 mimic and Lipofectamine 2000 reagent for 6 h, then LiCl (20 mmol l⁻¹) and/or AngII (1×10⁻⁶ mol l⁻¹) for 48 h; and (4) miR-126 inhibitor and Lipofectamine 2000 reagent (Invitrogen, Milan, Italy) for 6 h, then AngII (1×10⁻⁶ mol l⁻¹) for 48 h. The morphology of the cultured cells was observed by electron microscopy. Each treatment was performed at least 3 times.

RNA extraction and real-time quantitative PCR

Total RNA was extracted using the TRIzol method (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was reverse transcribed using Superscript III reverse transcriptase (Life Technologies, Carlsbad, CA USA) and miRNA primer. After reverse transcription, complementary DNA was subjected to quantitative PCR. MiRNA-specific looped primers were used. TaqMan micro-RNA assays (TaKaRa, Tokyo, Japan) for miR-221, miR-221, miR-126, miR-145, miR-21 and miR-210 were used for PCR amplification as described in the manufacturer's instructions. All reactions were performed in triplicate in a final volume of 10 μ l. Quantification was achieved after normalization using 18S ribosomal RNA values.

Western blot assay

A total of 20 μ g protein was separated on NuPAGE Novex 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA) and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% nonfat dry milk in phosphate-buffered saline containing 0.05% Tween for 1 h. After being washed with phosphate-buffered saline containing 0.05% Tween, the membrane was separately incubated with primary antibody overnight at 4°C and washed 4 times and incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h. Film was developed by chemiluminescence (PerkinElmer, Boston, MA, USA). Primary antibodies against rat Wnt4 (1:500), Dvl-1 (1:1000), β -catenin (1:500), COL1A1 (1:500) and COL3A1 (1:500) were used to detect the expression of protein in SMCs. Anti- β -actin (1:2000) was used to normalize loading variability.

MTT proliferation assay

The VSMC proliferation was determined by MTT assay. After incubation as described above, endothelial progenitor cells were digested with 0.25% trypsin and 0.02% EDTA. After centrifugation and resuspension in Dulbecco's modified Eagle's medium and 10% fetal bovine serum, 10^4 cells were cultured in serum-free medium in a 96-well culture plate (200 ml per well), whereas serum-free medium served as a control. After 72 h of culture, VSMCs were supplemented with 20 ml of MTT (5 g l^{-1}) and incubated for another 4 h. Then, the supernatant was discarded by aspiration, and the VSMCs preparation was shaken with 150 ml of Dimethylsulfoxide (DMSO) for 10 min before the optical density value was measured at 490 nm.

Cell treatment with miRNA inhibitor or mimics

VSMCs were treated with miR-126 mimic or miR-126 inhibitor (Ambion Pre-miR miRNA Precursors, Life Technologies, Ambion, Carlsbad, CA, USA) using Oligofectamine reagent (Life Technologies, Invitrogen, Milan, Italy) according to the manufacturer's instructions. MiRNA mimic negative control and miRNA inhibitor negative control served as negative controls in the respective experiments. Further analysis of the samples (infection or RNA isolation) was performed at 48 h after transfection. VSMCs were treated by indicated reagents after 6 h when pretreated with miR-126 mimics, miR-126 inhibitors or their negative controls.

Luciferase reporter assay

Prediction of miR-126 potential targets within the Wnt4 3' untranslated region (UTR) was performed with miRanda software

online (Target Scan: <http://www.targetscan.org/>, miRanda: <http://www.microrna.org/>, PITA: <http://genie.weizmann.ac.il/>). Fragments of 3'UTR of Wnt4 gene harboring the predicted miR-126-binding sites were cloned into the firefly luciferase reporter plasmid pMIR-Report (Ambion, Carlsbad, CA, USA) according to the manufacturer's protocol. The plasmid was transfected into VSMCs, and the cells were incubated for 24 h. After transfection, cells were harvested or lysed, and luciferase reporter activities were measured using the Dual-Glo Luciferase Assay System (Promega Corp., WI, USA). Firefly luciferase activity was normalized to *Renilla* luciferase activity and total protein determined using the bicinchoninic acid protein assay kit. Values for cells without miRNA mimic transfection were set equal to 1.

Statistical analysis

Numerical data were represented as mean \pm s.d. Continuous variables were tested for normal distribution with the Kolmogorov-Smirnov test. Differences between groups were assessed using one-way analysis of variance followed by the least significant difference test as a *post hoc* comparison. A value of $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) 16.0 software (IBM Corp, NY, USA) for Windows.

RESULTS

The establishment of a rat model of intimal thickening

Left carotid artery balloon injury was performed in rats. At 6 weeks after surgery, carotid arteries were removed, and integral and medial thickness were assessed by hematoxylin and

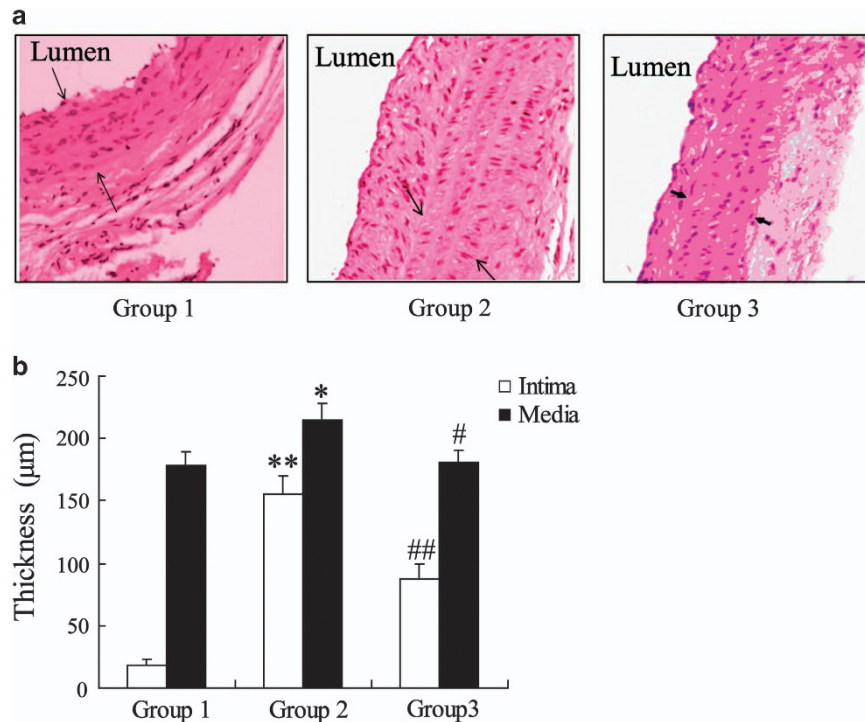


Figure 1 Emodin improved intimal hyperplasia in balloon-injured rat carotid artery. (a) Representation of the arterial vascular walls in three groups by using hematoxylin and eosin (H&E) staining (magnification $\times 200$; \(\backslash\) points to internal or external elastic plate; medial layer is the area between the two \(\backslash\)s; intima is the area next to internal elastic plate on the lumen side). (b) Statistical analysis of data on intimal and medial layer thickness of carotid artery in the three groups. Group 1, sham group; group 2, saline group; and group 3, emodin group. The data are represented as mean \pm s.d. * $P < 0.05$, ** $P < 0.01$ compared with sham group; # $P < 0.05$, ## $P < 0.01$ compared with the balloon injury group.

eosin staining. The results showed that injured carotid artery of the rats was markedly thickened, the intima was protruding into the albumen, cells and fibers were irregular and part of the internal elastic plate was damaged in the balloon injury group (Figure 1a). All morphologic features improved in rats that were administered emodin. Statistically, both the intima and media layers showed a thickening in the balloon injury group compared with the sham group. Because the thickness was partly reduced in rats that received emodin, it suggests an anti-restenosis effect of emodin (Figure 1b).

Emodin inhibited Wnt4/Dvl-1/ β -catenin signaling *in vivo* during intimal thickening

We tested expression of Wnt4 protein in injured carotid arteries using immunohistochemistry. Figure 2a shows that vascular injury enhanced expression of Wnt4 that was significantly attenuated by emodin treatment. Several miRNAs have been demonstrated to be related with arterial injury. Therefore, we next evaluated levels of various vascular injury-related miRNAs by real-time quantitative PCR. The results showed that compared with the sham group, vascular balloon injury altered expression levels of miR-221, miR-145, miR-21 and miR-210. Interestingly, only the level of miR-126 was regulated by emodin (Figure 2b).

To further assess the downstream signal of Wnt4 that mediates emodin-induced prevention of artery stenosis, we examined levels of Dvl-1 and β -catenin in injured arteries by using the western blot assay. The resulting data revealed that expression of Dvl-1 and β -catenin protein was activated by vascular balloon injury, but was inhibited in rats administered emodin (Figure 2c). In addition, expression of collagen-1 and collagen-3, which contributes to neointimal thickness, was also determined. As shown in the lower portion of Figure 2c, artery injury induced upregulation of collagen-1 and collagen-3 in the operation group, whereas the increase was reversed by emodin treatment, indicating a molecular basis for the anti-restenosis effect of emodin.

Emodin inhibited proliferation of VSMCs

To analyze the molecular mechanism underlying anti-restenosis effect of emodin, the experiment was performed in VSMCs. The proliferation of VSMCs was stimulated by AngII as previously performed. Similar to the *in vivo* experiment, VSMCs were pretreated with different doses of emodin for 24 h before exposure with AngII. Using MTT assay, we confirmed that emodin inhibited AngII-induced cell viability at 40 and 80 $\mu\text{mol l}^{-1}$ in a concentration-dependent manner (Figure 3a). Representation of the inverted microscope images showed that normally growing cells exhibited elongated spindles and that AngII clearly accelerated cellular growth (Figure 3b). However, proliferation of VSMCs was suppressed by emodin treatment in a concentration-dependent manner. These data indicated an antiproliferation effect of emodin in VSMCs.

Emodin inhibited levels of miR-126 and Wnt4/Dvl-1/ β -catenin signaling in VSMCs

As mentioned above, decreased expression of miR-126 after arterial injury was improved by emodin, indicating a regulatory role of miR-126 in emodin inhibition of intimal thickness. We therefore verified this pathway by testing emodin-induced inhibition of VSMC growth. As shown in Figure 4a, the expression of miR-126 was downregulated in VSMCs exposed to AngII, whereas the reverse was true in cells treated with emodin. Furthermore, proteins in the Wnt4/Dvl-1/ β -catenin signaling cascade were examined by western blot assay in VSMCs. Figure 4b shows an increase in expression of Wnt, Dvl and β -catenin proteins that were downregulated by emodin treatment. In addition, as shown in the lower portion of Figure 4b, AngII-induced upregulation of collagen-1 and collagen-3 was abrogated by emodin treatment, and this is in agreement with *in vivo* results.

Overexpression of miR-126 inhibited Wnt4/Dvl-1/ β -catenin signaling and collagen protein expression

To assess the possible regulatory role of miR-126 on the Wnt4/Dvl-1/ β -catenin signaling pathway in AngII-stimulated VSMCs, cells were treated with a miR-126 mimic that enhanced expression of miR-126 before exposure to AngII (Figure 5a). Prediction of potential miR-126 targets in the Wnt4 3'UTR was performed with miRanda software (data was not shown). At 48 h after miR-126 mimic transfection, Wnt4 3'UTR activity was significantly attenuated (Figure 5b) and expression of AngII-induced Wnt4/Dvl-1/ β -catenin signaling proteins was also inhibited (Figure 5c), indicating a negative regulatory effect of miR-126 on Wnt4/Dvl-1/ β -catenin signaling in VSMC proliferation. In addition, miR-126 overexpression suppressed AngII-induced expression of collagen-1 and collagen-3 in VSMCs, and this inhibition was reversed by LiCl (an agonist for Wnt4/ β -catenin signaling) (Figure 5d).

Silencing miR-126 abrogated the antagonistic effect of emodin on protein expression and growth of VSMCs

To further confirm the negative regulation correlation between miR-126 inhibition and the Wnt4/Dvl-1/ β -catenin signaling pathway during emodin treatment, miR-126 was knocked down by miR-126 transfection (Figure 6a) before VSMCs were exposed to AngII and or emodin, and signaling protein expression was evaluated. Wnt4 3'UTR activity was enhanced by silencing miR-126 (Figure 6b). The western blot results showed that emodin-inhibited expression of Wnt4/Dvl-1/ β -catenin proteins was abrogated by silencing miR-126 (Figure 6c). To test whether this signaling change had an effect on VSMC growth, cell proliferation was evaluated by MTT assay. As shown in Figure 6d, knockdown of miR-126 also reduced proliferation of cells when compared with the negative control group of emodin+AngII.

DISCUSSION

In this study, we provide the first direct evidence that emodin inhibited intimal and medial thickness *in vivo*. In addition,

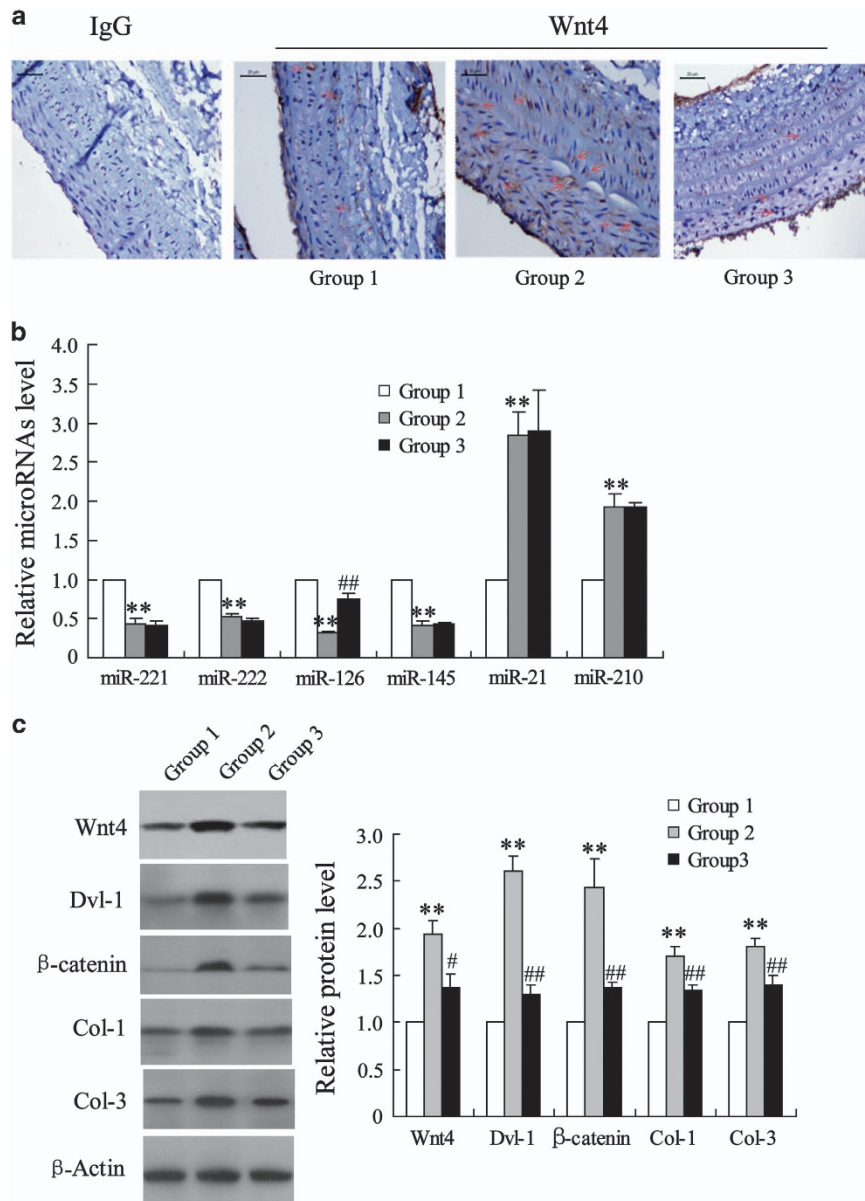


Figure 2 Emodin regulated expression of injury-induced signaling molecules and collagen in balloon-injured rat carotid artery. In the injured artery, (a) semiquantitative immunohistochemistry was used to determine Wnt4 protein expression; (b) real-time quantitative PCR (RT-Q-PCR) was used to analyze microRNA expression; and (c) western blotting was used to determine the Wnt/Dvl-1/β-catenin signaling proteins and collagen expression. Col-1, collagen-1; Col-2, collagen-2. The data are represented as mean ± s.d. ***P* < 0.01 compared with sham group; #*P* < 0.05, ##*P* < 0.01 compared with the balloon injury group.

we demonstrated that emodin improved restenosis via its inhibitory effect on VSMC proliferation. We then sought to verify the underlying mechanism of this effect, and the results indicated that emodin inhibited VSMC proliferation via inactivating the Wnt/Dvl-1/β-catenin signaling pathway via miR-126 upregulation.

The prevention of restenosis by emodin after artery injury is currently ill-defined. However, previous studies have indicated that emodin inhibited proliferation of VSMCs. It is well known that increased VSMC proliferation, migration and apoptosis rates radically alter the composition and structure of the blood vessel wall and contribute to restenosis

that occurs after PCI and stent implantation.²² Yin and Xu⁹ found that emodin has an antiproliferative effect on VSMC via blocking the transition of VSMC cell cycle from G0 to S after balloon endothelial denudation. It has also been demonstrated that emodin induces growth arrest and apoptosis of VSMCs.^{10,11} Although these studies imply that emodin exerts a negative effect on proliferation of VSMCs, they do not constitute direct evidence of an anti-restenosis effect of emodin. In this study, the use of balloon-injured carotid artery in rats provides the first direct proof that emodin improved intimal and medial thickness and was involved in preventing morphologic changes of the cells and fibers. A large amount of

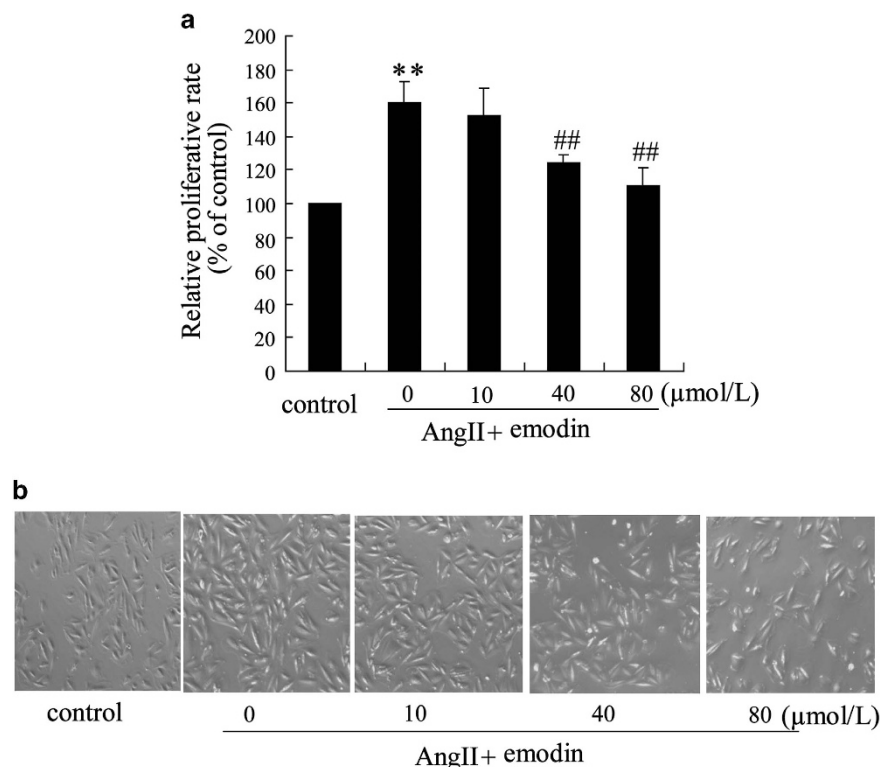


Figure 3 The effect of emodin on vascular smooth muscle cell (VSMC) growth. VSMCs were pretreated by emodin (10, 40 and 80 $\mu\text{mol l}^{-1}$) for 24 h and then incubated with angiotensin II (AngII; 1 $\mu\text{mol l}^{-1}$) for 48 h before further analysis. **(a)** Cell proliferation was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. **(b)** Cell growth state was observed by using inverted microscopy (magnification $\times 200$). The data are represented as mean \pm s.d. ** $P < 0.01$ compared with control; ## $P < 0.01$ compared with the balloon injury group.

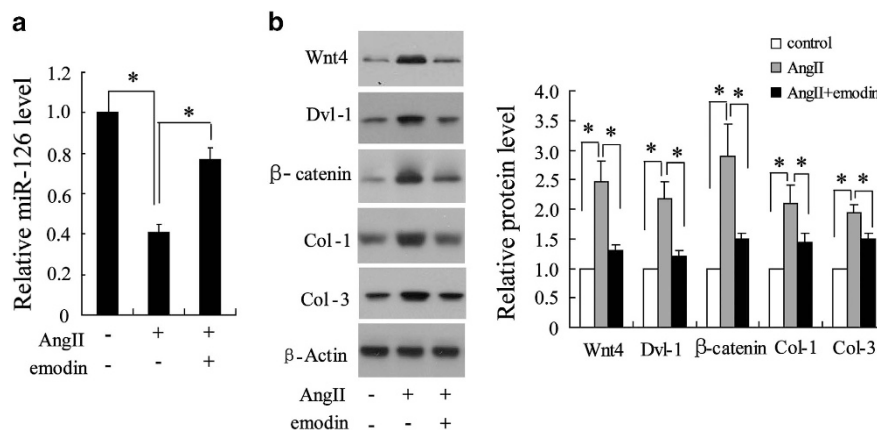


Figure 4 Emodin regulated angiotensin II (AngII)-induced expression of signaling molecules and collagen in vascular smooth muscle cells (VSMCs). VSMCs were pretreated with emodin (80 $\mu\text{mol l}^{-1}$) for 24 h and then incubated with AngII (1 $\mu\text{mol l}^{-1}$) for 48 h before further analysis. **(a)** The microRNA-126 (miR-126) expression was evaluated using real-time quantitative PCR (RT-Q-PCR). **(b)** Wnt4/Dvl-1/ β -catenin signaling protein and collagen protein expression was determined by western blotting. The data are represented as mean \pm s.d. * $P < 0.05$ compared with the corresponding group.

synthesis and secretion of collagen fibers in VSMCs is also a pathological reaction underlying restenosis after vascular injury.²³ The *in vivo* inhibition of collagen-1 and collagen-3 expression by emodin in VSMCs also further underlies its role in preventing restenosis.

Recent research has proposed that Wnt signaling is a novel regulator of VSMC proliferation and thereby involved in

intimal thickening. The important role of Wnt/ β -catenin signaling in SMC proliferation has been demonstrated by a number of important studies over the past decade.²² Tsaousi *et al.*¹⁴ showed that the Wnt/ β -catenin pathway is a critical modulator of arterial VSMC proliferation *in vitro* and contributes to pathological intimal thickening *in vivo*. Although these studies suggest the involvement of the Wnt/Dvl/ β -catenin

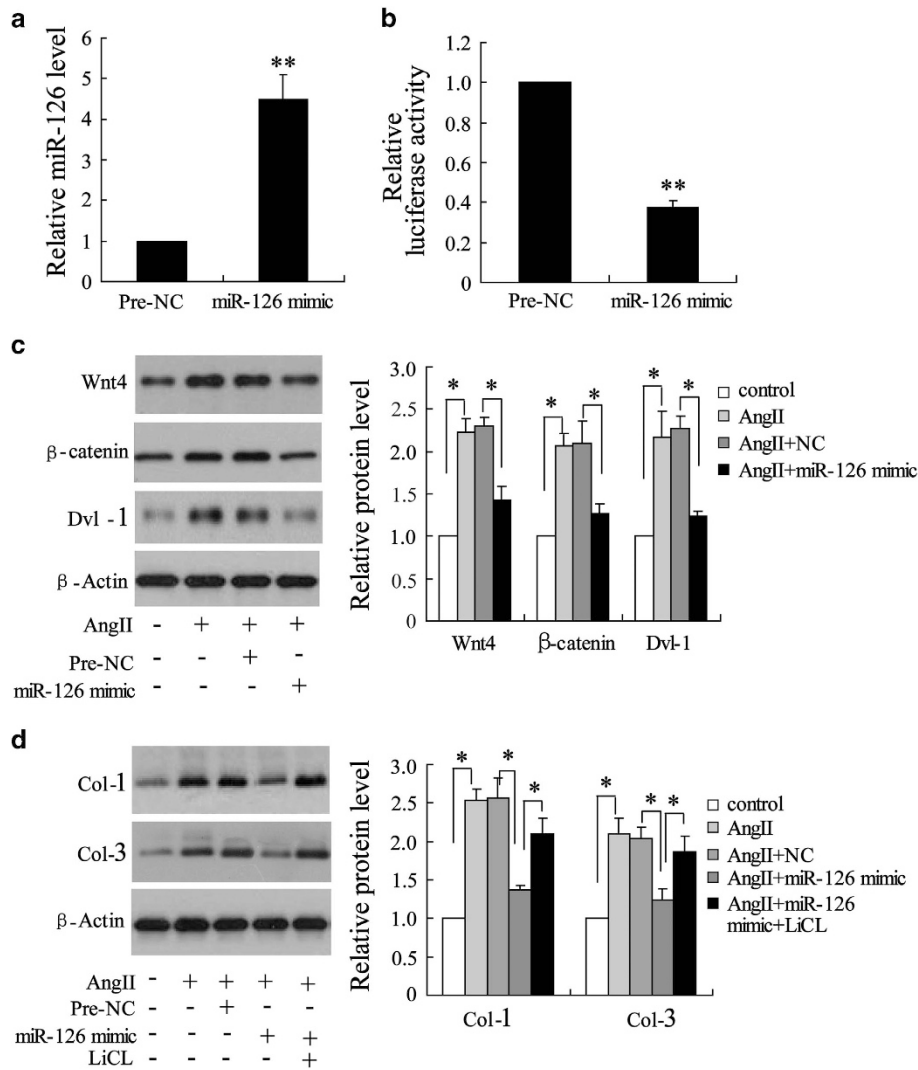


Figure 5 MicroRNA-126 (miR-126)-mediated Wnt4/Dvl-1/β-catenin signaling and collagen protein expression in angiotensin II (AngII)-stimulated vascular smooth muscle cells (VSMCs). The VSMCs were pretreated with miR-126 mimics (0.1 nmol l^{-1}) or negative control for 6 h and then incubated with or without AngII ($1 \mu\text{mol l}^{-1}$) and/or LiCl (20 mmol l^{-1}). The cells were lysed for the following analyses. (a) The miR-126 expression was determined by real-time quantitative PCR (RT-Q-PCR). (b) Wnt4 3' untranslated region (UTR) activity was evaluated using luciferase reporter assay. (c) Expression of Wnt4/Dvl-1/β-catenin signaling molecules and (d) collagen proteins was determined by western blotting. The data are represented as mean \pm s.d. ** $P < 0.05$ compared with negative group; * $P < 0.05$ compared with the corresponding group.

signaling pathway in VSMC proliferation and neointimal thickness, it is not clear whether this pathway is involved in anti-restenosis effect of emodin. In this study, we found that emodin inhibited Wnt/Dvl/β-catenin protein expression in rats after balloon-induced arterial injury, indicating a regulatory role of Wnt/Dvl/β-catenin signaling in emodin-induced improvement in intimal thickness.

In addition, our data showed that several arterial injury-related miRNAs were upregulated or downregulated in association with intimal thickness. Interestingly, only the level of miR-126 was regulated by emodin. MiR-126, an endothelial-specific miRNA, has recently been discovered to play a major role in vascular development and angiogenesis.^{16,17} Deletion of miR-126 causes a loss of vascular integrity and produces defects

in endothelial cell proliferation, migration and angiogenesis.⁹ Previous work has also revealed that miR-126 regulates Wnt/Dvl/β-catenin signaling in coxsackievirus replication.²⁴ However, no direct evidence was put forward about miR-126 regulation of Wnt/Dvl/β-catenin signaling in mammals. In this study, we predicted that Wnt4 3'UTR contained potential targets for miR-126 with miRanda software (data not shown). This was further demonstrated by Wnt4 3'UTR fluorescence activity assay. In Wnt/Dvl/β-catenin protein expression analysis, we demonstrated that upregulated miR-126 expression by a miR-126 mimic led to negative regulation of Wnt/Dvl/β-catenin signaling as well as collagen secretion. This effect was effectively reversed by LiCl (an agonist for Wnt/Dvl/β-catenin signaling) in VSMCs exposed to AngII. These findings suggest

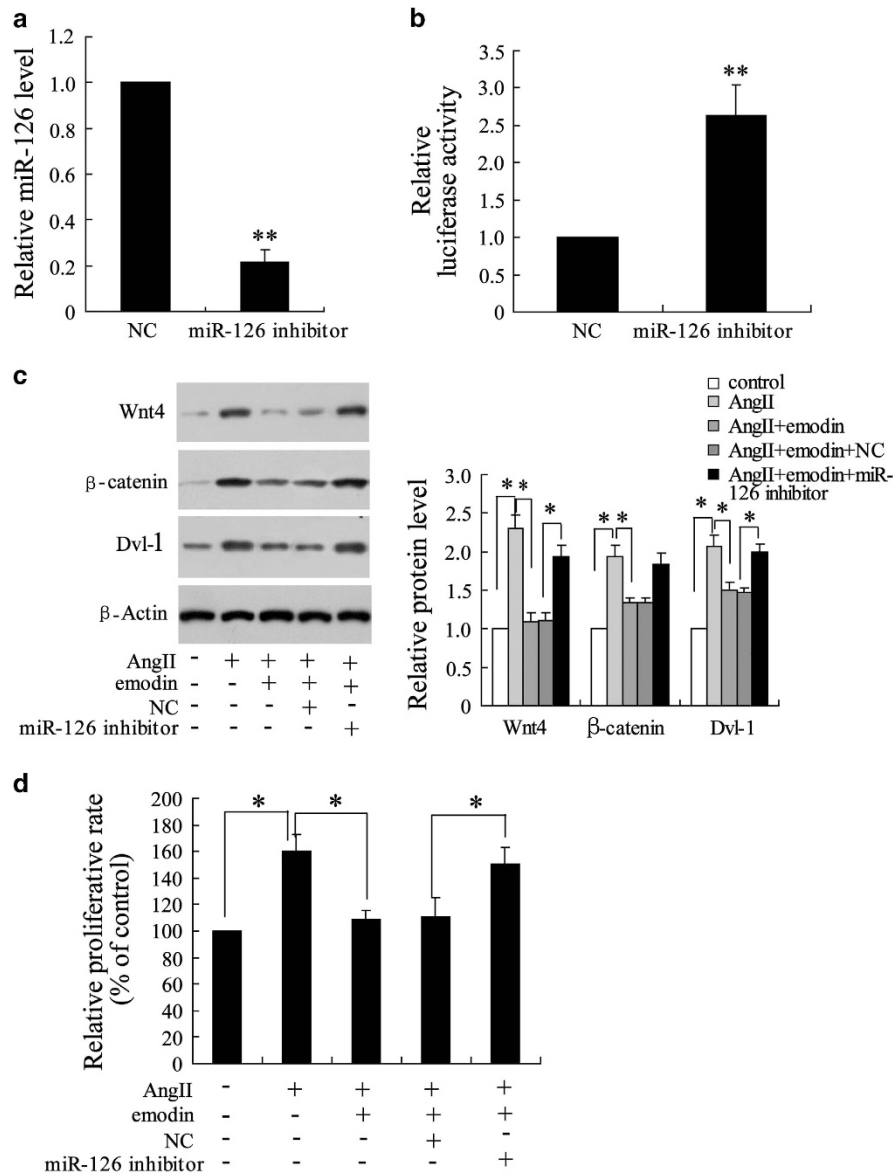


Figure 6 Knock down of microRNA-126 (miR-126) abrogated emodin-induced inhibition of cell proliferation in angiotensin II (AngII)-stimulated vascular smooth muscle cells (VSMCs). The VSMCs were pretreated with miR-126-inhibitor (0.1 nmol l^{-1}) or negative control for 6 h and then incubated with or without AngII ($1 \mu\text{mol l}^{-1}$) and/or emodin (80 nmol l^{-1}). The cells were lysed for the following analyses. **(a)** The miR-126 expression was determined using real-time quantitative PCR (RT-Q-PCR). **(b)** Wnt4 3' untranslated region (UTR) activity was evaluated by luciferase reporter assay. **(c)** Expression of Wnt4/Dvl-1/ β -catenin signaling molecules was determined with western blotting. **(d)** MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to determine cell proliferation. The data are represented as mean \pm s.d. ** $P < 0.05$ compared with negative group, * $P < 0.05$ compared with the corresponding group.

that the increase in miR-126 during VSMC proliferation contributes to the downregulation of Wnt/Dvl/ β -catenin signaling. To confirm this pathway, we investigated Wnt/Dvl/ β -catenin protein expression and cellular growth after knockdown of miR-126. The results show that the miR-126 inhibitor abrogated the inhibitory effect of emodin on Wnt/Dvl/ β -catenin signaling, and the decrease of cell viability induced by emodin was also reversed.

In summary, we identified that emodin can inhibit VSMC proliferation and has potential as an effective drug to prevent restenosis after arterial injury. Moreover, we found that

activation of miR-126 by emodin was a key mechanism by which emodin inhibits VSMC growth, and the regulation of Wnt/Dvl/ β -catenin signaling pathway by miR-126 is critical for the process. These findings enrich our understanding of the functional roles of emodin in arterial restenosis and provide novel insights into the development of therapeutic strategies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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Author contributions: Jun-yi Hua and Zhi-min Pan initiated and designed the study. Jun-yi Hua performed the statistical analysis and drafted the manuscript. Yun Xu, Yu-zhou He, Xu-hong Jiang and Wu Ye interpreted the data. Jun-yi Hua and Zhi-min Pan provided critical review of the manuscript and important intellectual content. All authors read and approved the final manuscript.

- 1 Stone GW, Ellis SG, Cox DA, Hermiller J, O'Shaughnessy C, Mann JT *et al*. A Polymer-based, paclitaxel-eluting stent in patients with coronary artery disease. *N Engl J Med* 2004; **350**: 221–231.
- 2 Ozer N, Tangurek B, Firat F, Ozer S, Tartan Z, Ozturk R *et al*. Effects of drug-eluting stents on systemic inflammatory response in patients with unstable angina pectoris undergoing percutaneous coronary intervention. *Heart Vessels* 2008; **23**: 75–82.
- 3 Patti G, Nusca A, Di Sciascio G. Meta-analysis comparison (nine trials) of outcomes with drug-eluting stents versus bare metal stents in patients with diabetes mellitus. *Am J Cardiol* 2008; **102**: 1328–1334.
- 4 Mnjoyan ZH, Doan D, Brandon JL, Felix K, Sitter CL, Rege AA *et al*. The critical role of the intrinsic VSMC proliferation and death programs in injury-induced neointimal hyperplasia. *Am J Physiol Heart Circ Physiol* 2008; **294**: H2276–H2284.
- 5 He L, Bi JJ, Guo Q, Yu Y, Ye XF. Effects of emodin extracted from Chinese herbs on proliferation of non-small cell lung cancer and underlying mechanisms. *Asian Pac J Cancer Prev* 2012; **13**: 1505–1510.
- 6 Lin SZ, Wei WT, Chen H, Chen KJ, Tong HF, Wang ZH *et al*. Antitumor activity of emodin against pancreatic cancer depends on its dual role: promotion of apoptosis and suppression of angiogenesis. *PLoS One* 2012; **7**: e42146.
- 7 Manu KA, Shanmugam MK, Ong TH, Subramaniam A, Siveen KS, Perumal E *et al*. Emodin suppresses migration and invasion through the modulation of CXCR4 expression in an orthotopic model of human hepatocellular carcinoma. *PLoS One* 2013; **8**: e57015.
- 8 Subramaniam A, Shanmugam MK, Ong TH, Li F, Perumal E, Chen L *et al*. Emodin inhibits growth and induces apoptosis in an orthotopic hepatocellular carcinoma model by blocking activation of STAT3. *Br J Pharmacol* 2013; **170**: 807–821.
- 9 Yin C, Xu C. Effect of aloe-emodin on proliferation of vascular smooth muscle cells after arterial injury. *Zhongguo Zhong Xi Yi Jie He Za Zhi* 1998; **18**: 420–422.
- 10 Wang X, Zou Y, Sun A, Xu D, Niu Y, Wang S *et al*. Emodin induces growth arrest and death of human vascular smooth muscle cells through reactive oxygen species and p53. *J Cardiovasc Pharmacol* 2007; **49**: 253–260.
- 11 Heo SK, Yun HJ, Park WH, Park SD. Emodin inhibits TNF-alpha-induced human aortic smooth-muscle cell proliferation via caspase- and mitochondrial-dependent apoptosis. *J Cell Biochem* 2008; **105**: 70–80.
- 12 Wang X, Adhikari N, Li Q, Hall JL. The LDL receptor related protein LRP6 regulates proliferation and survival through the Wnt cascade in vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* 2004; **287**: H2376–H2383.
- 13 Marchand A, Atassi F, Gaaya A, Leprince P, Le Feuvre C, Soubrier F *et al*. The Wnt/ beta-catenin pathway is activated during advanced arterial aging in humans. *Aging Cell* 2011; **10**: 220–232.
- 14 Tsaousi A, Williams H, Lyon C, Taylor V, Swain A, Johnson J *et al*. Wnt4/b-catenin signalling induces VSMC proliferation and is associated with intimal thickening. *Circ Res* 2011; **108**: 427–436.
- 15 Wang X, Xiao Y, Mou Y, Zhao Y, Blankesteijn M, Hall JL. A role for the b-catenin/ T-cell factor signaling cascade in vascular remodeling. *Circ Res* 2002; **90**: 340–347.
- 16 Uglow EB, Slater S, Sala-Newby GB, Aguilera-Garcia CM, Angelini GD, Newby AC *et al*. Dismantling of cadherin-mediated cell-cell contacts modulates smooth muscle proliferation. *Circ Res* 2003; **92**: 1314–1321.
- 17 Slater SC, Koutsouki E, Jackson CL, Bush RC, Angelini GD, Newby AC *et al*. R-cadherin:beta-catenin complex and its association with vascular smooth muscle cell proliferation. *Arterioscler Thromb Vasc Biol* 2004; **24**: 1204–1210.
- 18 Li PP, Wang X. Role of signaling pathways and miRNAs in chronic lymphocytic leukemia. *Chin Med J (Engl)* 2013; **126**: 4175–4182.
- 19 Caporali A, Emanuelli C. MicroRNA regulation in angiogenesis. *Vascuol Pharmacol* 2011; **55**: 79–86.
- 20 Meder B, Keller A, Vogel B, Haas J, Sedaghat-Hamedani F, Kayvanpour E *et al*. MicroRNA signatures in total peripheral blood as novel biomarkers for acute myocardial infarction. *Basic Res Cardiol* 2011; **106**: 13–23.
- 21 Taurino C, Miller WH, McBride MW, McClure JD, Khanin R, Moreno MU *et al*. Gene expression profiling in whole blood of patients with coronary artery disease. *Clin Sci (Lond)* 2010; **119**: 335–343.
- 22 Mill C, George SJ. Wnt signaling in smooth muscle cells and its role in cardiovascular disorders. *Cardiovasc Res* 2012; **95**: 233–240.
- 23 Osheroov AB, Gotha L, Cheema AN, Qiang B, Strauss BH. Proteins mediating collagen biosynthesis and accumulation in arterial repair: novel targets for anti-restenosis therapy. *Cardiovasc Res* 2011; **91**: 16–26.
- 24 Ye X, Hemida MG, Qiu Y, Hanson PJ, Zhang HM, Yang D. MiR-126 promotes coxsackievirus replication by mediating cross-talk of ERK1/2 and Wnt/beta-catenin signal pathways. *Cell Mol Life Sci* 2013; **70**: 4631–4644.



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