

Galectin-3 induces the phenotype transformation of human vascular smooth muscle cells via the canonical Wnt signaling

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Abstract. Galectin-3, a galactoside-binding protein, is highly expressed in carotid plaques and plays an important role in the atherosclerotic lesions. The phenotype transformation of vascular smooth muscle cells is the basic pathological change of atherosclerosis. This study investigated the effects of exogenous galectin-3 on the function and phenotype transformation of human umbilical vascular smooth muscle cells (HUSMC). In this study, we treated vascular smooth muscle cells with recombinant galectin-3 and tested its effect on cell proliferation, migration, and phenotype transformation. Our results showed that exogenous galectin-3 promoted human umbilical vascular smooth muscle cells (HUSMC) proliferation and migration. Exogenous galectin-3 enhanced the expression of the smooth muscle synthetic protein osteopontin, smooth muscle contractile proteins calponin and smooth muscle α -actin. The galectin-3-induced change in cell phenotype was associated with the activation of canonical Wnt signaling, as measured by β -catenin axin2 and cyclin D1 expression. β -catenin inhibition by small interfering RNA reduced cell proliferation, decreased cell motility, and blocked galectin-3-induced phenotype transformation of human umbilical vascular smooth muscle cells (HUSMC). Our data suggest galectin-3 promotes the phenotype transformation of human umbilical vascular smooth muscle cells (HUSMC) by activating Wnt/ β -catenin signaling pathway.

Introduction

Atherosclerosis is the primary cause of heart disease and stroke, and it is also one of the main causes of death and disability

around the world (1,2). The phenotype transformation of vascular smooth muscle cell (VSMC) plays an important role in the pathology process of atherosclerosis. In the atherosclerotic lesion, there is a large amount of the synthetic phenotype of VSMC in the walls of blood vessel. These synthetic phenotype VSMC tends to form foam-like cells and accelerate lesion progression (3,4). First, these cells secrete a variety of proteins (MMP-2, MMP-9, collagen type I and OPN), which increase lipid content and enhance the accumulation of monocytes and macrophages, second, they are believed to be activated because of enhancing ability of proliferation, immigration, and phagocytosis (3).

Galectin-3 (gal-3), a galactoside-binding protein, can be widely expressed by different kind of cells. Oxidized low-density lipoprotein (oxLDL) can also promote the expression of gal-3 in macrophages (5). In human umbilical vein endothelial cells (HUVEC), gal-3 plays an important role in vascular endothelial growth factor (VEGF)-and basic fibroblast growth factor (bFGF)-mediated angiogenesis (6). More importantly, Gal-3 is an important player in Aldo-induced vascular inflammation and mediates aldo-induced vascular fibrosis, silencing gal-3 blocks aldo-induced collagen type I deposition both *in vivo* and *in vitro* (7).

Gal-3 can regulate Wnt signaling (8). Gal-3 is a key regulator in the Wnt/ β -catenin signaling pathway and interact with axin-2, GSK-3 β and β -catenin immediately (9-11). Furthermore, gal-3 can in direct contact with β -catenin to stimulate cyclin D1 and c-myc expression (9). Tatsuo even find that human gal-3 sequence has a structural similarity to β -catenin in the breast cancer cells (10).

Recently, the role of gal-3 in cardiovascular disease has also been widely studied by some researchers (12-17). In the setting of atherosclerotic disease, gal-3 seems to promote atherogenesis. Gal-3 is an useful biomarker which can predict the subsequent infarction after first myocardial infarction (MI) (18). Furthermore, in the absence of gal-3, the development of atherosclerotic pathological change in the ApoE-deficient mouse is reduced (19). Patients with type 2 diabetes and arterial hypertension have higher levels of gal-3 in plasma (20). Gal-3 has been proved to be a promising biomarker for detecting prediabetes and diabetes (21). Until now the mechanism of gal-3 in atherogenesis is still not clear yet, we presumed that gal-3 was a high risk factors of atherosclerosis and could

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promote pathological process of atherosclerosis. Considering the importance of phenotype transformation of SMC, does gal-3 induce atherosclerosis development by modulate cellular phenotype? If it does, what is the mechanism involved?

Thus, in this study, we tried to investigate the effects of exogenous gal-3 in HUSMCs. Furthermore, we examined whether β -catenin, a reported signaling associated with phenotype transformation of vascular smooth muscle cells, is involved in this gal-3 activity.

Materials and methods

Reagents. The Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin (pen/strep, 10,000 U/ml each) were purchased from Gibco (Carlsbad, CA, USA). oxLDL was obtained from Peking Union-Biology Co., Ltd. (Beijing China). TRIzol reagent for RNA isolation was purchased from Invitrogen (Carlsbad, CA, USA). XAV939 was purchased from Sigma-Aldrich (St. Louis, MO, USA). The reverse transcriptase kit (RR037A) and SYBR Premix Ex Taq (DRR420S) were purchased from Takara (Dalian China). The Cell Counting Kit-8 (CCK-8) assay kit was purchased from Dojindo (Kumamoto, Japan). Recombinant human gal-3 was purchased from PeproTech (Rocky Hill, NJ, USA). The primary antibody against gal-3 (no. sc-20157), SMA (no. sc-32251), cyclin D1 (no. sc-8396) and GAPDH (no. sc-48166) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-phospho GSK3 β (Ser9) (no. CST-9322), anti-GSK3 β (no. CST-9315), anti-nonphospho (active) β -catenin (no. CST-4270), and anti- β -catenin (no. CST-9582) were acquired from Cell Signalling Technology, Inc. (Danvers, MA, USA). The antibody against osteopontin (OPN; no. ab91655), calponin (no. ab46794) and axin2 (no. ab109307) were obtained from Abcam (Cambridge, UK). The goat anti-rabbit secondary antibody (no. A-21109) and the goat anti-mouse secondary antibody (no. A-21058) were provided by Invitrogen. All other chemicals were from commercial sources.

Cells culture. A primary culture of human SMCs was established by explant outgrowth of a segment of human umbilical cord retrieved at the time of caesarean section (22). Endothelial cells were removed by scraping the luminal surface of the vessel with a cotton swab, and the adventitia was mechanically stripped away. Primary cultures were maintained in DMEM supplemented with 20% FBS and 1% antibiotics (penicillin/streptomycin) (all from Gibco). Cells between 4th and 10th passages were used in these experiments. The trial confirmed with the principle of the Declaration of Helsinki and was approved by the Ethics Committee of the Shanghai Ninth Hospital.

siRNA interference. β -catenin expression was inhibited by transfection with a siRNA specific to β -catenin. β -catenin siRNA was transiently transfected into the cells using Lipofectamine[®] 2000 (Invitrogen Life Technologies), according to the manufacturer's instructions. Briefly, 5×10^5 HUSMCs per well were cultured in 6-well plates to 75% confluence. The cells were then transfected with 100 pmol siRNA duplexes using 5 μ l Lipofectamine[®] 2000 and 500 μ l DMEM (reduced

serum medium). The process of transfection was without antibiotics. Following a 72-h incubation at 37°C, the cells were harvested for analysis. The human β -catenin siRNA sequence was 5'-CATGUGUTGGUAAAGCUCUA-3' and the scrambled siRNA sequence was 5'-GCAACAGTTGCAGAGAGGU-3'. They were synthesized by Biotend (Shanghai, China).

Cell proliferation assay. Cell proliferation was measured with the CCK-8 assay kit. In brief, 5,000 cells were plated in each well of a 96-well plate and allowed to attach for 24 h. Then, HUSMCs were subsequently incubated for 0, 12, 24 or 48 h in the presence of gal-3. Subsequently, the plate was incubated with CCK-8 for 4 h at 37°C. At last, the absorbance of wave length was taken at 450 nm.

Migration assay. The assay was performed as previously described (23). Migration assays were performed in a chamber. HUSMCs were resuspended in 200 μ l serum-free DMEM medium and 5×10^4 HUSMCs were loaded into the upper chambers. The lower chamber was filled with 400 μ l of DMEM in the presence or absence of 10 μ g/ml recombinant gal-3. The chamber was incubated at 37°C for 24 h. Then, the lower side of the filter was washed with PBS and fixed with 4% paraformaldehyde. Nuclei were stained with 4',6-diamino-2-phenylindole (DAPI, 1:1,000; Sigma) for 5 min at room temperature. The cells were counted in three random high-power fields (x100) in each well.

Real-time RT-PCR analysis. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA was reverse-transcribed into cDNA using reverse transcriptase, and quantitative real-time PCR was performed using SYBR-Green master mix, on an Applied Biosystems 7500 real-time PCR system, according to the manufacturer's instructions. Specific primers for human gal-3, β -catenin, OPN, axin2, calponin, SMA and GAPDH were as follows: Gal-3 forward, 5'-GGCCACTGATTGTGCCTTAT-3' and reverse, 5'-TGCAACCTTGAAGTGGTCAG-3'; β -catenin forward, 5'-GCCGGCTATTGTAGAAGCTG-3' and reverse, 5'-GAGTCCCAAGGAGACCTTCC-3'; OPN forward, 5'-TGA GTCTGGAAATAACTAATGTGTTTGA-3' and reverse, 5'-GAACATAGACATAACCCTGAAGCTTTT-3'; axin2 forward, 5'-CTCTCTACCTCATTTCCCGAGAAC-3' and reverse, 5'-CGAGATCAGCTCAGCTGCAA-3'; calponin forward, 5'-ATGTGAGGAGGGAAGAGTGTG-3' and reverse, 5'-CGGTGGAAGTGAGCAGAGG-3'; SMA forward, 5'-AGC GTGGCTACTCCTTCGTGAC-3' and reverse, 5'-GCTCGTT GCCGATGGTGATGAC-3'; cyclin D1 forward, 5'-AATGACC CCGCAGGATTC-3' and reverse, 5'-TCAGGTTTCAGGCCT TGCAC-3'; GAPDH forward, 5'-TGATGACATCAAGAAGG TGGTGAAG-3' and reverse, 5'-TCCTTGGAGGCCATGTGG GCCAT-3'.

Western blot analysis. Cells were lysed in a lysis buffer containing 150 mM of NaCl, 10 mM of Tris (pH 7.5), 5 mM of EDTA, 1% Triton X-100, 1 mM of PMSF, 10 mg/ml of leupeptin, 10 mg/ml of pepstatin, and 10 mg/ml of aprotinin for 30 min on ice. The nuclear and cytoplasmic protein were separately extracted by using the kit (P 0028; Beyotime Institute of Biotechnology (Shanghai, China). Protein concentrations were

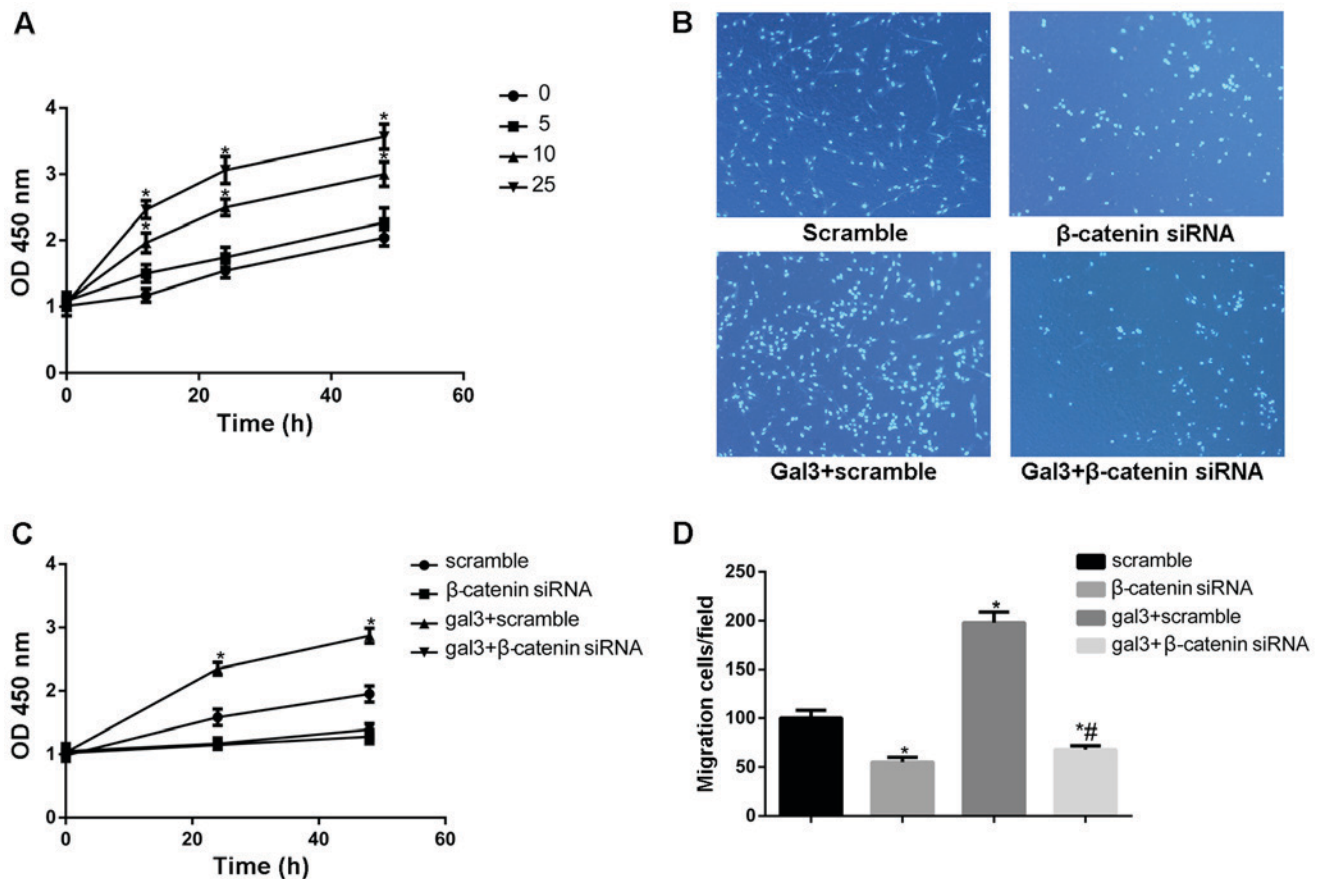


Figure 1. Exogenous gal-3 promotes HUSMCs proliferation and migration. Proliferation assays. Cells were treated with gal-3 over a range of concentration (0-25 $\mu\text{g/ml}$), CCK-8 kit was added at different time-points to detect cell proliferation (A). Native HUSMCs were defined as control. * $P < 0.05$ vs. control. After transfection with either control- or β -catenin-siRNA for 24 h, HUSMCs were incubated for 48 h in the absence or presence of gal-3 (25 $\mu\text{g/ml}$). CCK-8 were used to detect cell proliferation (C). Cells transfected with scramble siRNA were defined as control. * $P < 0.05$ vs. control. Migration assays. Cells migrated to the lower surface of each chamber are shown. After transfection with either control or β -catenin siRNA for 24 h, migration was tested by a Transwell method. Migrated cells that migrated from the upper chamber to the lower chamber were counted in five nonoverlapping fields under a microscope ($\times 10$) (B and D). Bars are represented as the mean \pm SD ($n = 5$). * $P < 0.05$ vs. control; # $P < 0.05$ vs. gal-3. Data (mean \pm SD) were obtained from three independent experiments.

measured with the BCA Protein Assay (Pierce Biotechnology Inc., Rockford, IL, USA). The lysates (20 μg) were electrophoresed on 10% SDS-PAGE and transferred to nitrocellulose membranes (Merck Millipore, Danvers, MA, USA). The membrane was blocked with 5% nonfat dry milk in TBST buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 0.1% Tween-20) for 1 h at room temperature. The blots were then incubated with various 1000-fold diluted primary antibodies at a dilution of 1:1,000 in TBST at 4°C overnight, and then washed twice with TBST buffer at room temperature and incubated for 1 h with the appropriate peroxidase-conjugated secondary antibody (1:5,000 dilution). All signals were detected by Odyssey (LI-COR Biosciences, Lincoln, NE, USA). To quantify the protein, band intensity was assessed by Quantity One 4.6.2 software.

Statistical analysis. All data are expressed as the mean \pm SD. Statistics were performed using the SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). One-way ANOVA followed by the Student-Newman-Keuls post hoc analyses were used. For data that not passed the normality test; non-parametric ANOVA (Kruskal-Wallis test) was used. A value of $P < 0.05$ was considered statistically significant. All experiments were performed at least three times.

Results

Exogenous galectin-3 promotes HUSMCs proliferation and migration. First, we used increasing concentrations of gal-3 (up to 25 $\mu\text{g/ml}$) to stimulate cells. Cell proliferation was determined at 12, 24, and 48 h using an CCK-8 assay. As shown in Fig. 1A, the proliferation of HUSMCs was significantly increased in a concentration dependent manner. The Transwell migration assay was used to investigate the effect of recombinant gal-3 on cell migration. As shown in Fig. 1B and C, exogenous gal-3 enhanced HUSMCs migration significantly.

Galectin-3 promotes the phenotype transformation of vascular smooth muscle cells. We also investigated the role of exogenous gal-3 in the phenotype transformation of HUSMC. First of all, the HUSMCs were cultured in the DMEM without serum for 12 h. After that, the serum-starved HUSMCs were treated with gal-3 for 48 h. As shown in the Fig. 2, the expression of the smooth muscle synthetic proteins, OPN rose significantly over a range of gal-3 concentration (0-25 $\mu\text{g/ml}$). Concomitant with the increased OPN expression, the protein markers of contractile phenotype were also increased in gal-3-treated cells, as measured by SMA and calponin expression.

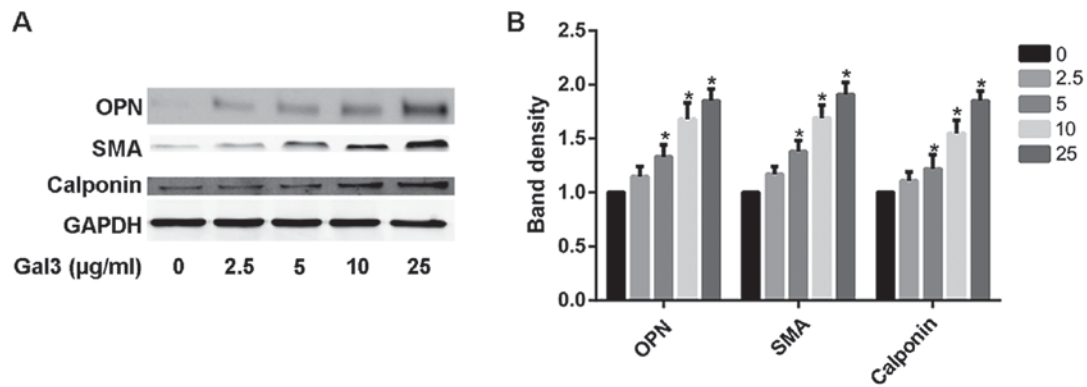


Figure 2. Gal-3 promotes the phenotype transformation of vascular smooth muscle cells. Cells were treated with gal-3 for 48 h over a range of concentration (0-25 $\mu\text{g/ml}$), and the expression of OPN, SMA, and calponin were measured by western blotting (A). Respectively densitometric measurement results are given (B). GAPDH protein expression was monitored as controls. Band density of native HUSMCs were defined as control and set to 1, * $P < 0.05$ compared with control. Data (mean \pm SD) were obtained from three independent experiments.

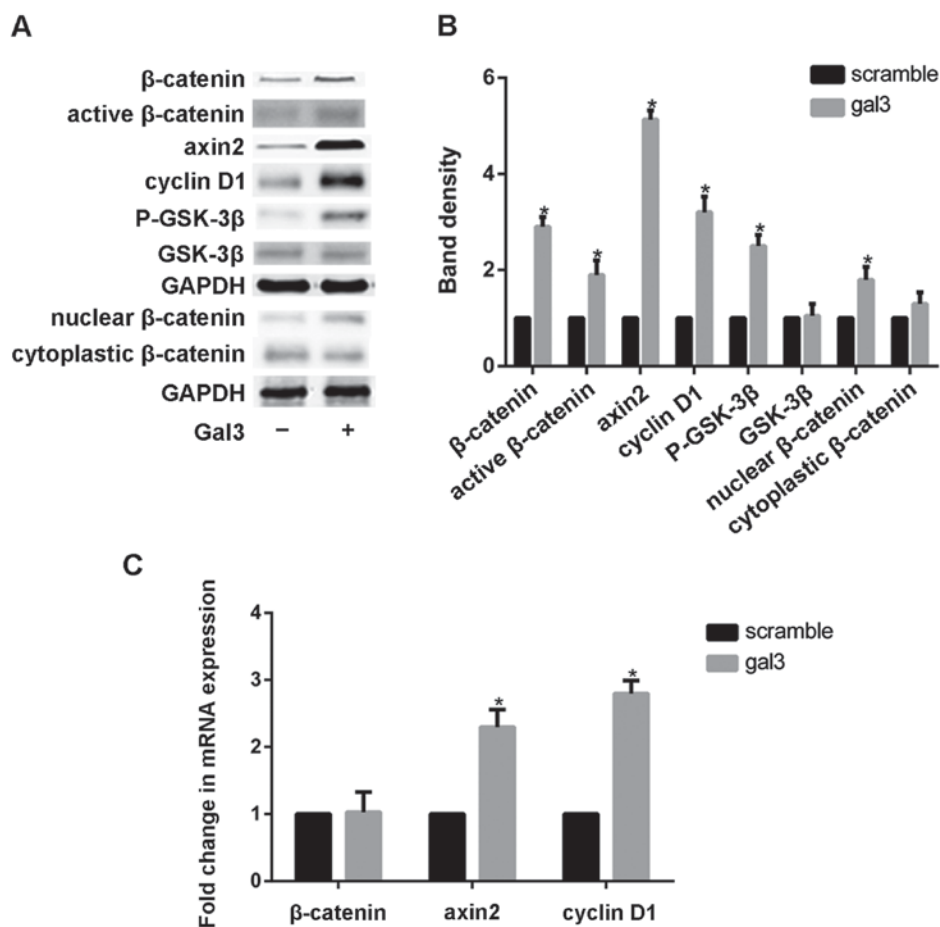


Figure 3. Gal-3 activates canonical Wnt signaling in human umbilical vascular smooth muscle cells. Cells were treated with 25 $\mu\text{g/ml}$ gal-3 for 48 h, and the expression of β -catenin, cyclin D1 and axin2 were measured by western blotting and real-time PCR (A-C). GAPDH protein expression was monitored as controls. GSK-3 β , p-GSK-3 β , nonphosphorylated β -catenin, nuclear β -catenin and cytoplasmic β -catenin were measured by western blotting. Band density of native HUSMCs was defined as control and set to 1, * $P < 0.05$ compared with control. Data (mean \pm SD) were obtained from three independent experiments.

Galectin-3 activates canonical Wnt signaling in human umbilical vascular smooth muscle cells. Canonical Wnt signal activation could be evaluated by recording β -catenin protein levels (24). The expression of β -catenin was tested by western blotting after cells were treated with gal-3. The distribution of β -catenin in cytoplasm and nucleus were examined by western blot analysis separately. Gal3 obviously increased

the expression of β -catenin in nucleus, but it has relatively little effect on the cytoplasmic β -catenin. To further confirm the activation of canonical Wnt signaling by gal-3, we also detected the expression of axin2 (regulating β -catenin stability) (25) and cyclin D1 (β -catenin target gene) by using real-time-PCR and western blotting. Our results showed that recombinant gal-3 significantly increased expression of axin2

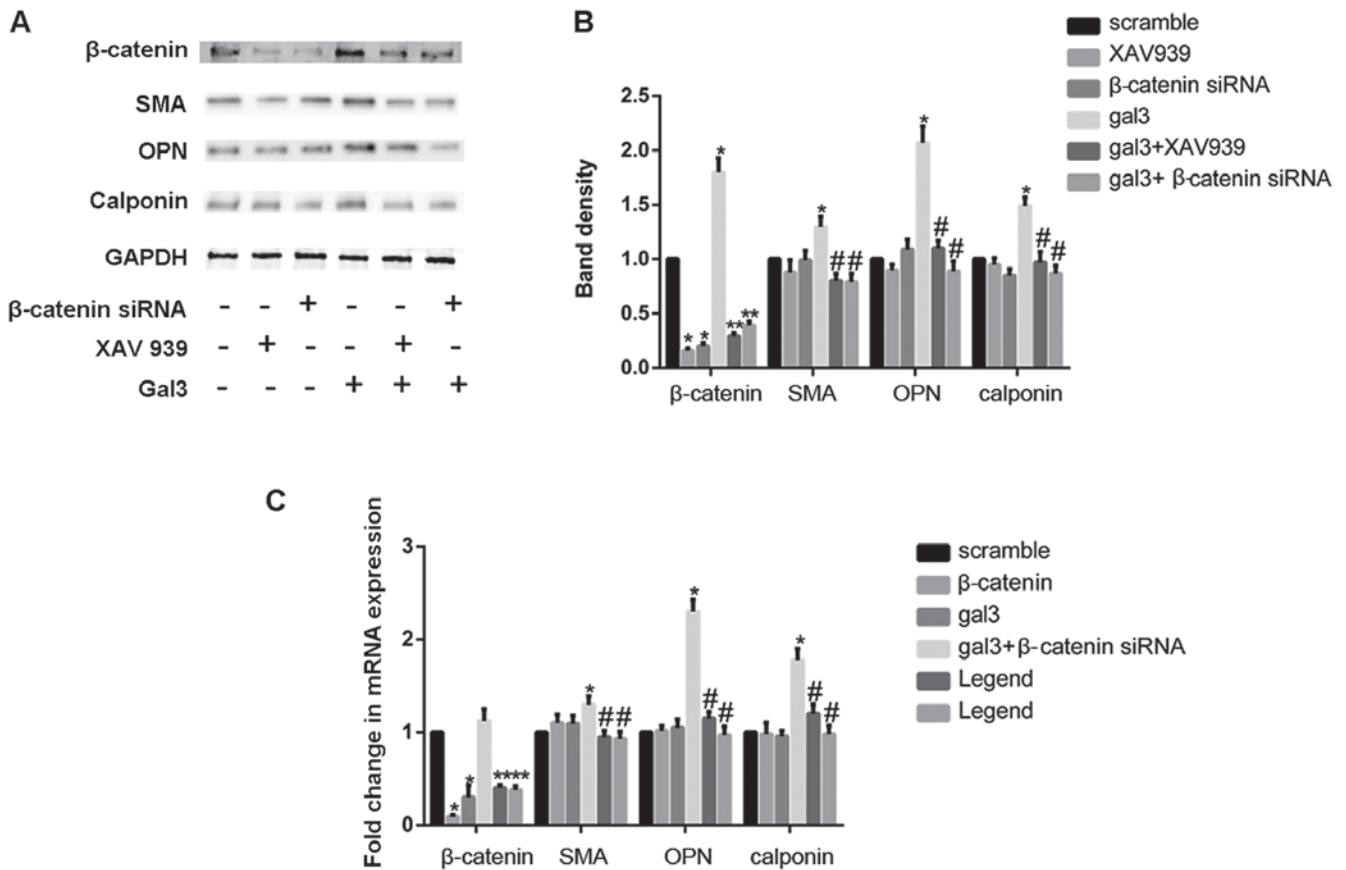


Figure 4. Canonical Wnt/β-catenin signaling mediates the effects of gal-3 in HUSMCs. (1) After transfection with either control or β-catenin siRNA for 24 h, HUSMCs were incubated for 48 h in the absence or presence of gal-3 (25 μg/ml) or XAV939 (10 μM), the expression of β-catenin, OPN, SMA, and calponin were measured by realtime-PCR and Western blot. Realtime-PCR and Western blot results of β-catenin, OPN, SMA, and calponin are shown (A and B). Respectively densitometric measurement results are given (C). The GAPDH expression was used for protein level normalization. Band density of HUSMCs which was transfected with scramble siRNA was defined as control and set to 1. *P<0.05 compared with control. #P<0.05 compared with gal-3. ****P<0.05 compared with gal-3 and control. Data (means ± SD) were obtained from three independent experiments.

and cyclin D1 (Fig. 3). Gal-3 induced a marked increase in the protein expression of nonphosphorylated (active) and total β-catenin. However, the mRNA level of β-catenin was almost unchanged in the gal-3-treated cells (Fig. 3C). In order to confirm that β-catenin expression was modulated by gal-3 at the post-translational level, we also detected the activity of GSK-3β which promotes the degradation of β-catenin. Phosphorylation GSK-3β was 2.5-fold increased by gal-3, while total GSK-3β were almost unchanged.

Canonical Wnt/β-catenin signaling mediates the effects of galectin-3 in HUSMCs. To further evaluate the role of β-catenin in gal-3-mediated change of HUSMC, the expression of β-catenin was inhibited by siRNA strategy in HUSMCs. The siRNA of β-catenin reduced the expression of β-catenin mRNA and protein by 91 and 83%, respectively. These results indicated that it effectively antagonized the activation of Wnt/β-catenin signaling pathway. We then preformed cell proliferation and migration assays on these cells, the silencing of β-catenin decreased the proliferation and migration of HUSMCs (Fig. 1B and D). Furthermore, the increased ability of migration and proliferation in gal-3-treated cells were also eliminated after silencing of β-catenin. We then detected the role of β-catenin in the phenotypic switch induced by gal-3. β-catenin knockdown efficiently blocked the increase

expression of OPN, SMA and calponin induced by gal-3 in both mRNA and protein levels, as demonstrated by western blot and realtime PCR. In order to further confirm the role the Wnt signaling pathway in gal-3 induced phenotype transformation of VSMC, XAV939, a inhibitor of Wnt signaling pathway, was also used. XAV939 similarly attenuated the effect of gal-3 in HUSMCs (Fig. 4A-C).

Discussion

In this study, we demonstrate, for the first time, that gal-3 can activate the canonical Wnt signaling pathway in HUSMCs. More importantly, we find that gal-3 promote the phenotype transformation of HUSMCs, including increasing proliferation and migration and upregulating a series of phenotype related proteins. While using the strategy of β-catenin siRNA can obviously block the gal-3 induces phenotype transformation of HUSMCs. Taken all these data together, we conclude that gal-3 promotes the activation and phenotype transformation of HUSMCs through canonical Wnt/β-catenin signaling.

Gal-3 has been reported to affect the function of almost all the cells in the wall of blood vessel. In human umbilical vein endothelial cells (HUVEC), gal-3 is a mediator of vascular endothelial growth factor (VEGF)- and basic fibroblast

growth factor (bFGF)-mediated angiogenesis (6,26). Gal-3 can also induce the migration of macrophage in mouse (27). Atherosclerosis is characterized by the activation and accumulation of smooth muscle cells in the intimal layer of blood vessels where they internalized a lot of lipids (28). The enhanced proliferation and migration of smooth muscle cells are the symbol of the early pathology of atherosclerosis. In this study, we also showed that exogenous gal-3 can obviously promote the proliferation and migration of HUSMCs. In order to further explore the role of gal-3 in the HUSMCs, we also detected the expression of some widely used SMC marker proteins.

Traditionally, smooth muscle cells are thought of being exist in two totally different phenotypes: Contractile and synthetic phenotype. It is now being recognized that there are a diversity of SMC phenotypes, ranging from contractile to synthetic (4,29,30). In fact, these so-called contractile and synthetic phenotypes should be envisaged as 'idealized' phenotypes. Higher growth rates and stronger migratory activity are usually considered typical of the 'synthetic' phenotype, and OPN is also identified as a synthetic-related protein (3). Actually, recent studies proved that different synthetic and contractile markers could be upregulated at the same time (4,26). In some instances, contractile differentiation can be up-regulated in the 'synthetic' phenotype and contractile differentiation markers may express with matrix synthesis (28,31). In our study, we found that gal-3 stimulated the expression of OPN. Interesting, at the same time, gal-3 also increased the contractile-related protein, calponin and SMA. Therefore, we believe that galectin-3 can promote expression of both smooth muscle synthetic and contractile proteins.

There are two different subclasses of Wnt signalling: The non-canonical pathway (which is β -catenin dependent) and the canonical pathway (β -catenin independent). The activation of non-canonical Wnt pathway can involve the intra-cytoplasmic release of Ca^{2+} and the activation of Jun N-terminal kinase (JNK). The canonical Wnt signalling pathway involves the inhibition of β -catenin degradation complex, therefore resulting in the translocation of β -catenin from the cytoplasm to the nucleus, and binding to downstream target genes. Earlier reports have shown that β -catenin can regulate the expression of OPN, SMA and calponin expression, induce cell proliferation and migration in many types of cells (28,32). Recently, the role of gal-3 in vascular smooth muscle cells osteogenic differentiation has also been confirmed (32). Thus, we explored the role of β -catenin in gal-3-treated HUSMCs. In our study, gal-3 activated the canonical Wnt signaling pathway by upregulating the total and nonphosphorylated β -catenin. We also found that the expression of β -catenin downstream gene *axin2* and *cyclin D1* was upregulated by gal-3 (28,32). It further indicated that Wnt/ β -catenin signaling pathway was activated by gal-3 in the HUSMCs. Our results showed that gal-3 induced the expression of total β -catenin at protein level but not mRNA level. It is consensus with previous findings. Gal-3, a binding of β -catenin, increases phosphorylation of GSK-3 β (12,33,34). Inactivation of GSK-3 β can reduce the degradation of β -catenin, and increase cellular β -catenin levels besides, we found that gal-3 indeed increased the phosphorylated level of GSK-3 β . In order to prove the role of β -catenin in gal-3-induced phenotype transformation of HUSMCs, we

used the siRNA strategy and XAV939 to decrease the expression of β -catenin. Our results demonstrated that silencing of β -catenin inhibited gal-3-induced protein expression and blocked gal-3-mediated cell proliferation and migration of vascular smooth muscle cells. Thus, we conclude that recombinant gal-3 induces phenotype transformation of HUSMCs via canonical Wnt/ β -catenin pathway.

In conclusion, our results show that gal-3 promotes the activation and the phenotype transformation in HUSMCs. This activity is through the canonical Wnt signaling pathway. Recently, gal-3 has been proved to be related to the atherosclerosis and coronary heart disease. As smooth muscle cells plays an important role in a variety of plausible mechanisms of atherosclerosis and chronic heart diseases. Our research could help to clarify the role of gal-3 in the pathological process of atherosclerosis.

Acknowledgements

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