Wnt3a activates β1-integrin and regulates migration and adhesion of vascular smooth muscle cells

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Abstract. Vascular smooth muscle cells (VSMCs) are known to undergo functional changes that contribute to the pathogenesis of atherosclerosis and restenosis. Wnts are a family of secreted glycoproteins that bind to transmembrane Frizzled receptors and initiate signaling cascades with indispensable roles during cell migration, adhesion, proliferation, and survival. The present study reports that wingless-type MMTV integration site family, member 3a (Wnt3a) activates the canonical Wnt pathway in rat VSMCs by triggering the phosphorylation of β-catenin at position Ser675 and GSK-3β at position Ser9. Phosphorylation of these two proteins increases VSMC migration and adhesion. In a search for the downstream mediators of Wnt3a's effects on VSMC migration and adhesion, Wnt3a treatment was observed to increase integrin-linked kinase (ILK) protein expression. ILK is a serine/threonine protein kinase that is thought to control cell migration and adhesion by regulating the affinity of β1-integrin for the extracellular matrix. Wnt3a treatment of VSMCs also activated β1-integrin without changing the quantity of protein expressed on the cell surface. These results demonstrate that Wnt3a enhances migration and adhesion of VSMCs by activating β1-integrin.

Introduction

Signaling by members of the wingless-type MMTV integration site (Wnt) family of proteins controls various cellular and biological processes, ranging from cell adhesion, cell migration, and cancer development to differentiation of multiple cell lineages, cell polarity, and stem cell self-renewal (1). The majority of research on Wnts has focused on the Wnt/β-catenin pathway, previously referred to as the canonical Wnt pathway. Activation of the canonical Wnt pathway leads to nuclear translocation of β-catenin, which binds to lymphoid enhancer-binding factor/T cell-specific transcription factor (LEF/TCF) and activates transcription of target genes (1-3). The stability of β-catenin is also regulated by integrin-linked kinase (ILK), a serine/threonine protein kinase that interacts with the cytoplasmic domains of β1 and β3 integrins. Integrins composed of α and β transmembrane subunits are bidirectional signaling molecules. Inside-out signaling by integrins occurs when direct interactions between the cytoplasmic tail of β-subunits and the cytoskeletal protein, talin, regulate the affinity for the ligand. Following ligand binding, integrins engage in outside-in signaling by recruiting signaling and adaptor proteins to the cytoplasmic tail of the α and/or β subunits. This recruitment results in actin reorganization and modulation of various intracellular signaling pathways (4,5). ILK-integrin interactions organize the connections of the extracellular matrix (ECM) to the cytoskeleton and, by doing so, regulate cell migration and adhesion. In addition, ILK activity links integrin function to Wnt signaling (6-9). At present, *in vitro* and *in vivo* evidence supports the hypothesis that phosphorylation by ILK stimulates β-catenin to translocate into the nucleus and form a complex with LEF. In addition, ILK phosphorylates and inactivates glycogen synthase kinase-3β (GSK-3β). Inactivation of GSK-3β eventually results in activation of canonical Wnt target genes (6-10). However, little is known about how Wnt/β-catenin signaling activates ILK or integrin signaling. In the current study, we activated the canonical Wnt signaling by recombinant Wnt3a to investigate the migration and adhesion ability of vascular smooth muscle cells (VSMCs) and the role of ILK and β1-integrin.

Materials and methods

Cell culture and reagents. Primary VSMCs were isolated from the thoracic aorta of male Sprague Dawley rats (weight, 100-150 g), and then cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Beyotime, Shanghai, China). The cells were incubated at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. The purity of the VSMCs was estimated to be ~90%,

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based on cell morphology and the results of immunostaining with monoclonal mouse anti-α-actin antibodies (Sigma-Aldrich, St. Louis, MO, USA). The VSMCs used for all experiments were between the 3rd and 6th passages. Recombinant Wnt3a was purchased from R&D Systems (Minneapolis, MN, USA). In this study, anti-β-catenin antibody (polyclonal, rabbit) and anti-phospho-β-catenin (Ser675) antibody (polyclonal, rabbit; both from Cell Signaling Technology Inc., Danvers, MA, USA), anti-GSK-3β antibody (polyclonal, rabbit), anti-phospho-GSK-3β (Ser9) antibody (polyclonal, goat) and anti-β-actin antibody (polyclonal, rabbit; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-ILK antibody (polyclonal, rabbit; Sigma-Aldrich, St. Louis, MO, USA), anti-β1-integrin antibody (monoclonal, rabbit) and anti-active-β1-integrin antibody (monoclonal, mouse; both from Millipore, Billerica, MA, USA) were used.

Transwell migration assay. The VSMC migration assay was performed using transwell cell culture inserts (Transwell Assay system; Corning Inc., Acton, MA, USA) for 24-well plates. A total of 100 μl VSMCs ($3x10^5$ cells/ml) suspended in serum-free DMEM was added to the upper polycarbonate membrane insert (pore size, 8 μm), Wnt3a (final concentration, 100 ng/ml) was added to the upper chamber and 600 μl culture medium containing 10% FBS was added to the lower chamber. The cells were allowed to migrate for 24 h while the plates were incubated in a humidified incubator in a 5% CO_2 atmosphere at 37˚C. After 24 h, the cells that remained on the upper surface of the membrane were removed with a cotton swab. The membrane was fixed with anhydrous methanol for 20 min at room temperature and then stained with 0.1% crystal violet for 15 min. A microscope (Nikon, Tokyo, Japan) was used to determine the number of migratory cells by counting the cells in five randomly selected fields of view. All experiments were performed in triplicate.

Wound healing assay. VSMCs were plated in 6-well plates and grown to 70-80% confluency. The experimental wounds were created by dragging 200 μl pipette tips across the bottom of the cell culture wells. The cells were rinsed with phosphate-buffered saline (PBS) and the culture medium was replaced with fresh maintenance medium supplemented with Wnt3a (final concentration; 100 ng/ml). The wound healing was recorded after 48 h using bright field microscopy. The wound gap was measured and the percentage of wound repair was determined.

Cell adhesion assay. The cell adhesion assay was performed as described previously (11,12). Ninety-six-well tissue culture plates (Costar, Corning Inc.) were coated overnight at 4˚C with collagen type I (Sigma-Aldrich; 20 μg/ml). After washing with PBS, the plates were blocked with PBS containing 1% heat-denatured bovine serum albumin for 1 h at room temperature. The plates were washed extensively with serum-free DMEM, and $3x10^4$ VSMCs were plated and incubated with Wnt3a (final concentration, 100 ng/ml) for 1 h at 37˚C. After washing with PBS, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature and stained with 0.5% toluidine blue for 15 min. The plates were washed extensively with double distilled water. A microscope (Nikon) was used to determine the number of adherent cells by counting the cells in five randomly selected fields of view. The cells were solubilized in 1% sodium dodecyl sulfate (SDS) and quantified using a microtiter plate reader set at 590 nm (Tecan, Männedorf, Switzerland).

Western blot analysis. VSMCs were harvested following treatment with Wnt3a (final concentration; 100 ng/ml) or PBS (control) for three days. The cells were lysed by incubation with radioimmunoprecipitation (RIPA) buffer supplemented with proteinase inhibitors for 30 min on ice. Equal quantities of protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and subjected to immunoblot analysis according to standard protocols. The separated proteins were blotted onto nitrocellulose membranes and incubated with antibodies that recognize total β-catenin, phospho-β-catenin (Ser675), total GSK-3β, phospho-GSK-3β (Ser9), β1-integrin, ILK and β-actin overnight at 4˚C. Following three washes, the blots were incubated with peroxidase-conjugated secondary antibodies (Boster, Wuhan, China) for 1 h at room temperature and subsequently analyzed using an enhanced chemiluminescence detection system (Clinx, Shanghai, China).

Flow cytometric analysis. VSMCs were serum starved for 24 h and supplemented with Wnt3a (final concentration, 100 ng/ml) for three days. β1-integrin expression on the surface of the VSMCs was evaluated by using indirect immunofluorescence and flow cytometry. Following washing with PBS, the cells were incubated with a rabbit anti-rat β1-integrin antibody (dilution; 1:70) and a mouse anti-rat active β1-integrin antibody (dilution; 1:200) for 30 min at room temperature in the dark. The cells were washed again and incubated with phycoerythrin-conjugated goat anti-rabbit IgG (dilution, 1:50; Bioss, Beijing, China) and Cy3-conjugated goat anti-mouse IgG (dilution, 1:50; Bioss) secondary antibodies for 45 min. Following incubation with secondary antibodies, the cells were analyzed by flow cytometry with a Becton-Dickinson FACS Calibur and Cell Quest software (BD, Franklin Lakes, NJ, USA).

Statistical analysis. Paired or independent t-tests were performed for statistical analyses of all data. $P<0.05$ was considered to indicate a statistically significant difference. The data are expressed as the mean ± standard error of the mean.

Results

Wnt3a promotes VSMC migration. Transwell migration and wound healing assays were performed to investigate the effect of Wnt3a treatment on VSMC migration. The migratory ability of VSMCs treated with Wnt3a was significantly increased in the transwell migration assay (Fig. 1A). Following 24 h incubation, the number of cells that migrated across the polycarbonate membrane was higher in the Wnt3a group compared with the control group ($P<0.05$). As shown in Fig. 1B, VSMCs treated with Wnt3a moved faster than the control cells. These results demonstrate that Wnt3a treatment induces VSMC migration.

Adhesion of VSMCs to collagen type I. To investigate the influence of Wnt3a on cell-matrix interactions, adhesion assays were performed. Cells were stained with toluidine blue and the microplate reader method was used to monitor cell adhesion. The results demonstrated that the number of VSMCs

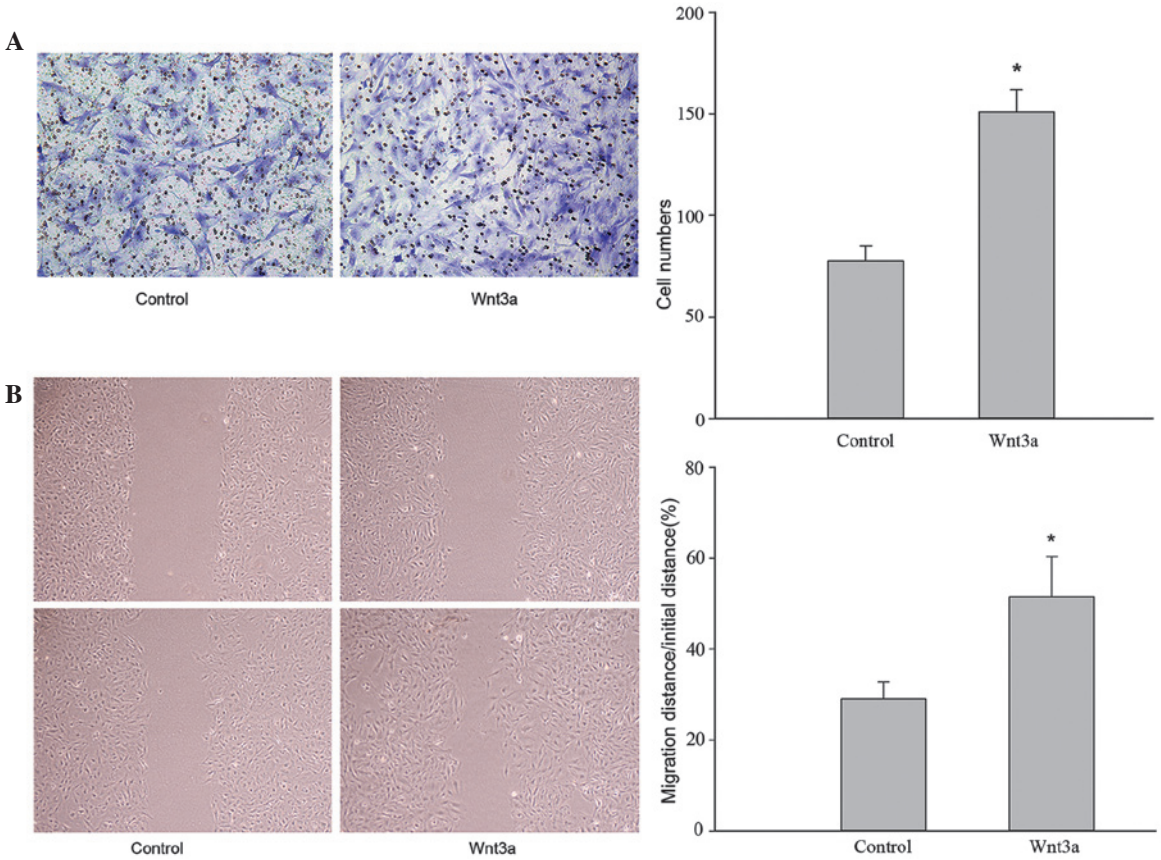

Figure 1. Wnt3a promotes VSMC migration. (A) Transwell migration assay. VSMCs on transwell inserts were stained with 0.1% crystal violet (left panel; magnification, x200). The number of migratory VSMCs was significantly higher in the Wnt3a-treated group compared with the phosphate-buffered saline-treated group (right panel; *P<0.05 vs. control). (B) A wound healing assay demonstrated that treatment with Wnt3a increased the migratory ability of VSMCs (left panel; magnification, x40). The migration distance of VSMCs treated with Wnt3a was significantly higher compared with control cells (right panel; *P<0.05, vs. control). VSMCs, vascular smooth muscle cells; Wnt3a, wingless-type MMTV integration site family, member 3a.

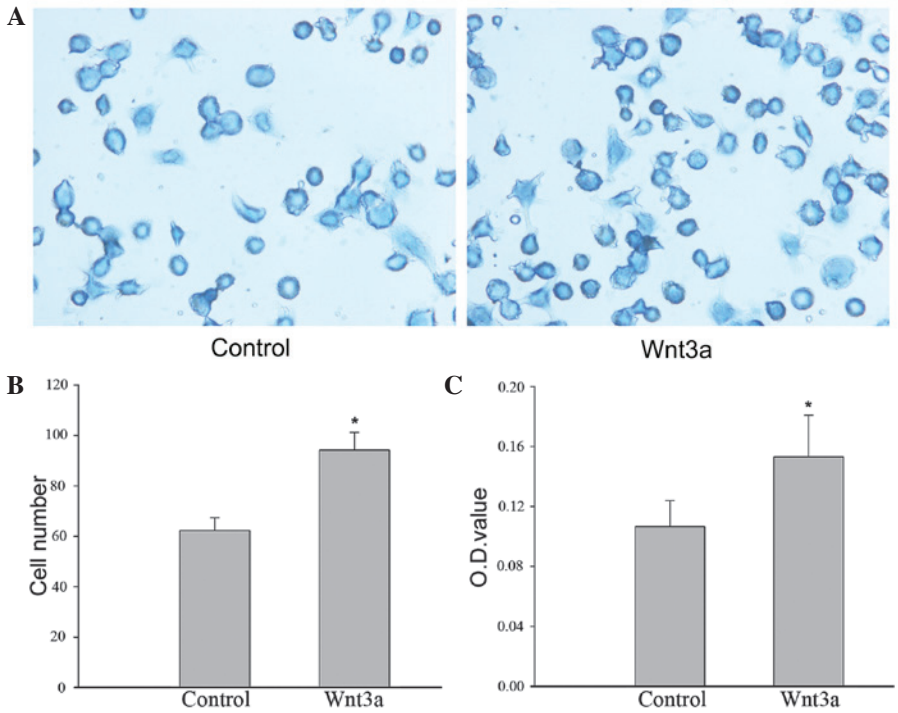

Figure 2. VSMC adhesion to collagen type I. (A) VSMCs that were cultured on plates were stained with toluidine blue (magnification, x400). (A and B) Wnt3a treatment increased VSMC adhesion to collagen type I. Cell adhesion was estimated by measuring the absorbance at 590 nm. (C) The optical density value was higher in samples from the Wnt3a-treated group than in those from the control group *P<0.05, vs. control. VSMCs, vascular smooth muscle cells; Wnt3a, wingless-type MMTV integration site family, member 3a.

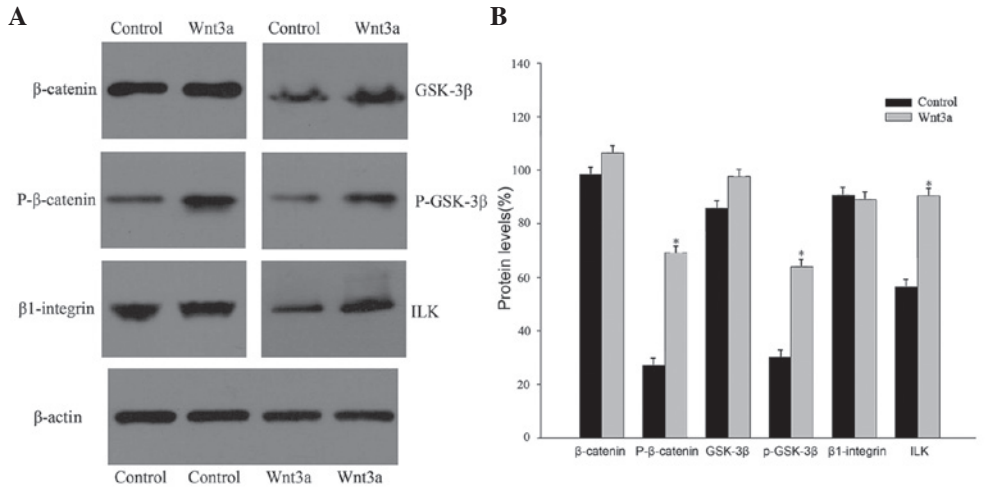

Figure 3. Protein expression was measured in cultured rat VSMCs that were treated with recombinant Wnt3a or phosphate-buffered saline (control) for three days. (A) Western blot analysis showing total β-catenin, phospho-β-catenin (Ser675), total GSK-3β, phospho-GSK-3β (Ser9), β1-integrin, and ILK protein expression in control and Wnt3a-treated cells. (B) The phospho-β-catenin and phospho-GSK-3β protein signals were normalized to those of total β-catenin and total GSK-3β, respectively. The β1-integrin and ILK protein signals were quantified and normalized to that of β-actin (*P<0.05, vs. control). GSK-3β, glycogen synthase kinase 3β; ILK, integrin-linked kinase; P-, phosphorylated; VSMCs, vascular smooth muscle cells; Wnt3a, wingless-type MMTV integration site family, member 3a.

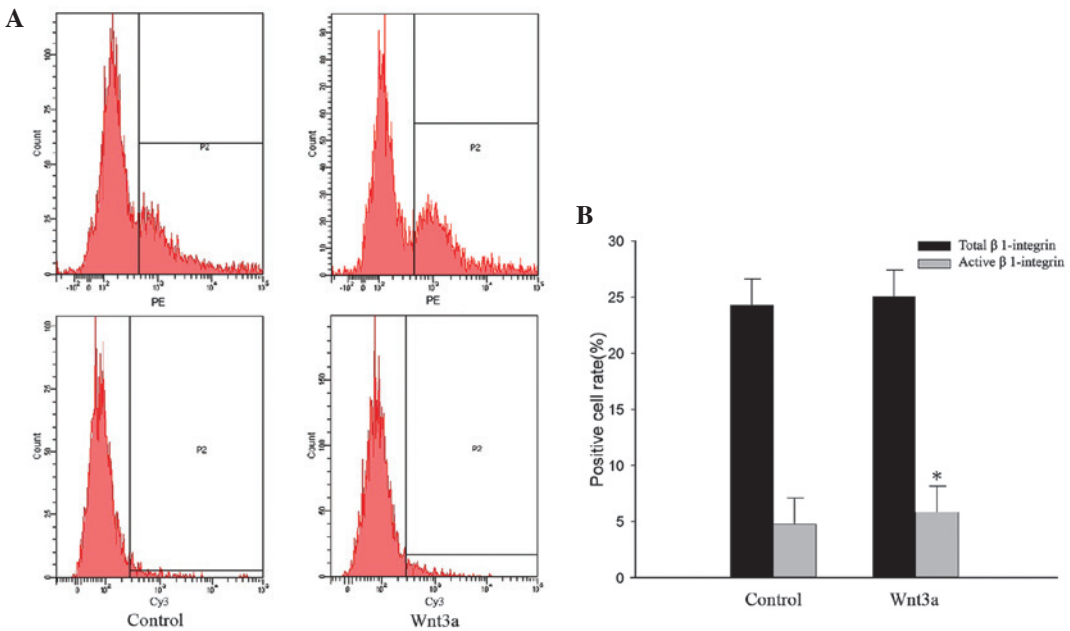

Figure 4. Wnt3a activates β1-integrin. (A) Active β1-integrin and total β1-integrin expression on the surface of VSMCs was evaluated by flow cytometry. (B) Treatment with Wnt3a activated β1-integrin but did not affect the expression levels on the cell surface (*P<0.05, vs. control). VSMCs, vascular smooth muscle cells; Wnt3a, wingless-type MMTV integration site family, member 3a.

that adhered to collagen type I was significantly greater in the Wnt3a-treated group compared with the control group. In addition, the optical density value detected by the microplate reader was higher in the samples from the Wnt3a-treated group compared with those from the control group (Fig. 2). These results indicate that Wnt3a treatment significantly improves the adhesion of VSMCs to collagen type I.

Wnt3a regulates protein expression. Wnt3a is a prominent member of the Wnt family and may induce the accumulation of β-catenin and activate the canonical Wnt pathway (13). To understand the mechanisms by which Wnt3a affects VSMCs, the protein expression of β-catenin, GSK-3β, ILK, and β1-integrin were analyzed, all of which are either components of, or targets of the canonical Wnt signaling pathway. Treatment of VSMCs with Wnt3a upregulated the expression of phospho-β-catenin (Ser675), phospho-GSK-3β (Ser9), and ILK; however, the expression of total β-catenin, total GSK-3β, and β1-integrin was not significantly different between the two groups (Fig. 3).

Wnt3a activates β1-integrin. Since treatment with Wnt3a did not alter the expression of β1-integrin, the expression of active β1-integrin on the surface of VSMCs was quantified using flow

cytometry (Fig. 4A). The anti-active-β1-integrin antibody is specific for the active conformation of rat β1-integrin, and it may also discriminate between the activated states. Therefore, it is useful to investigate how β1-integrin activation is regulated. VSMCs that were stimulated with Wnt3a for three days bound more active-β1-integrin antibodies compared with the unstimulated cells. However, Wnt3a treatment had no effect on the total quantity of β1-integrin expression on the surface of VSMCs. These results demonstrate that Wnt3a treatment activates β1-integrin without changing its expression levels (Fig. 4).

Discussion

It is well known that the Wnt signaling pathways affect migration and adhesion via downstream effectors (14-18). The canonical Wnt pathway controls cell migration and adhesion by regulating the stability of β-catenin, the major downstream target of Wnt signaling (14-18). Previous studies have determined a multitude of points of crosstalk between the Wnt pathways and the mechanisms that control cellular architecture, from the level of receptors to the level of transcription. Cellular mechanisms that are responsible for the regulation of migration and adhesion also function to modulate the activity of several Wnt pathway components (14,19,20). Activation of β-catenin signaling may occur by inhibition of GSK-3β and GSK-3β-mediated phosphorylation of residues in the N-terminal region of β-catenin may lead to rapid degradation. However, β-catenin is also activated by phosphorylation of serine 675, in the C-terminal region of the protein. This phosphorylation facilitates the nuclear translocation of β-catenin and enhances its transcriptional activity, as has been shown to occur in VSMCs *in vitro* upon activation by v-akt murine thymoma viral oncogene homolog 1 (Akt) or cAMP-dependent protein kinase, catalytic subunit C α (PKA) (20-23). Enhanced β-catenin phosphorylation at Ser675 and enhanced GSK-3β phosphorylation at Ser9 in rat VSMCs were observed following Wnt3a treatment. Phosphorylation of GSK-3β at Ser9 may inactivate the protein. Therefore, although a role of PKA, Akt, or other signaling pathways in β-catenin phosphorylation may not be excluded, the current data suggest that stimulation of VSMCs with Wnt3a activates the Wnt/β-catenin pathway.

VSMC migration and proliferation contribute to arterial wound repair and thickening of the intimal layer in atherosclerosis and restenosis. These processes are influenced by cell adhesion to molecules present in the ECM and are regulated by the integrin family of cell-surface matrix receptors. An important signaling molecule acting downstream of integrin receptors is ILK. ILK has been implicated in the control of cancer cell growth and survival through modulation of downstream targets, notably Akt and GSK-3β (24-26). Evidence also exists to establish ILK as a molecular adaptor protein linking integrins to the actin cytoskeleton and regulating actin polymerization (8,24-27). *In vitro*, ILK may phosphorylate and inactivate GSK-3β to promote the nuclear accumulation of β-catenin and activation of the canonical Wnt pathway (6-10). However, it remains unclear how activation of the canonical Wnt pathway influences the activity of ILK. In the current study, the role of the canonical Wnt pathway in ILK activation was investigated by culturing rat VSMCs with recombinant Wnt3a, and assessing ILK protein expression and β1-integrin activity. The results demonstrate that Wnt3a, not only activates the canonical Wnt pathway, but also increases ILK protein expression and β1-integrin activity.

The present study also demonstrates that treatment with Wnt3a significantly increases VSMC migration and adhesion. Cell adhesion to the ECM via integrins triggers the assembly of the actin cytoskeleton and regulatory proteins that form a large multiprotein complex. Through their association with the actin cytoskeleton and signaling molecules, adhesion complexes generate elaborate networks that control a variety of cellular processes in normal and pathological conditions, including cell migration, proliferation, survival, and also invasion and metastasis (28-31). Adhesion of VSMCs to collagen type I has been shown to be β1-integrin-dependent (11,32). Although adhesion of VSMCs to collagen type I is predominantly mediated via β1-integrin receptors, the cell surface expression levels of β1-integrin were not altered by Wnt3a stimulation (33). As demonstrated by flow cytometry, Wnt3a treatment activated β1-integrin in rat VSMCs. The mechanism by which intracellular signals alter the affinity and avidity of integrin receptors, termed 'inside-out' signaling, has been characterized by several groups (4,5,34). ILK is known to bind to the cytoplasmic domain of integrin subunits. This binding either increases affinity by triggering conformational changes in the integrin subunits or increases avidity by stimulating integrin clustering in the cell membrane (8,24-27). The results of the current study lead to the conclusion that Wnt3a activates ILK and β1-integrin, which then regulates VSMC migration and adhesion.

In conclusion, the Wnt/β-catenin pathway in rat VSMCs has been observed to activate β1-integrin without leading to quantitative changes in its expression on the cell surface. ILK binds to β1-integrin to increase the affinity of β1-integrin for the ECM by inside-out signaling, which may increase cell migration and adhesion. Wnt3a has been observed to regulate the migration and adhesion of VSMCs, and may therefore be a valuable therapeutic target for the prevention and treatment of vascular disease. However, vascular disease is a complicated process that is modulated by multiple signaling pathways. Future studies should investigate the mechanisms and functions of Wnt3a signaling in other cell lines and model systems.

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