

# Kindlin-2 siRNA inhibits vascular smooth muscle cell proliferation, migration and intimal hyperplasia via Wnt signaling

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**Abstract.** It is known that vascular smooth muscle cell (VSMC) proliferation and migration leads to intimal hyperplasia in cases of atherosclerosis and restenosis. In the present study, we investigated the effects of kindlin-2 on VSMC proliferation, migration and intimal hyperplasia, and the underlying mechanisms. The left common carotid artery of Sprague-Dawley rats were subjected to balloon injury in order to induce intimal hyperplasia, and then transfected with kindlin-2 small interfering RNA (siRNA) lentivirus or negative control siRNA lentivirus. We noted that the degree of intimal hyperplasia 4 weeks after balloon injury was significantly reduced in arteries transfected with kindlin-2 siRNA lentivirus ( $P < 0.05$ ). *In vitro*, kindlin-2 siRNA suppressed VSMC proliferation and migration induced by Wnt3a (100 ng/ml). Western blot analyses and RT-qPCR revealed that kindlin-2 regulated Wnt/ $\beta$ -catenin signaling and thereby modulated the expression of  $\beta$ -catenin target genes, including c-myc and cyclin D1. This study demonstrated that kindlin-2 plays a critical role in VSMC proliferation, migration and intimal hyperplasia via Wnt signaling. Therefore, blocking the activity of kindlin-2 represents a novel therapeutic strategy for vascular injury.

## Introduction

Vascular smooth muscle cell (VSMC) proliferation, migration and cell-extracellular matrix (ECM) adhesion are all known to contribute to the development of intimal hyperplasia in certain vascular pathologies, including restenosis and atherosclerosis (1,2). Interactions between VSMCs and the ECM regulate these processes through binding of the integrin family of cell adhesion receptors (2). A key mediator of integrin signaling

is kindlin-2, a recently discovered family of evolutionarily conserved four point one protein, ezrin, radixin and moesin (FERM) domain-containing proteins that binds directly to the cytoplasmic tails of  $\beta 1$  and  $\beta 3$  integrins (3-5). Kindlin-2 is essential for integrin clustering and activation, and regulates cell adhesion and directed migration by guiding the formation and maturation of focal adhesions (FA) and the organization of the cytoskeleton (6). Due to its essential role in integrin activation, kindlin-2 is involved in many important physiological processes, including heart development, cell migration and cancer progression (4,7). In mice, the loss of kindlin-2 causes peri-implantation lethality resulting from detachment of the endoderm and epiblast from the basement membrane as a consequence of diminished levels of  $\beta 1$ -integrin and also of  $\beta 1$ -integrin activation (8). In zebrafish, knockdown of the kindlin-2 gene resulted in severe abnormalities in the development of the heart. Ultrastructural analysis has revealed disrupted intercalated disc formation and that myofibrils failed to attach to the membrane complexes (9). Even partial inactivation of the kindlin-2 gene markedly impairs angiogenesis in mice and zebrafish, which arises from defective activation of integrin  $\alpha V \beta 3$  (10). In *in vitro* experiments using cells derived from kindlin-2 deficient mice or small interfering RNA (siRNA)-knockdown mice, defects in integrin activation despite the presence of talin were noted (10). Recently, Yu *et al* found that kindlin-2 forms a transcriptional complex with  $\beta$ -catenin and transcription factor 4 (TCF4) and enhances Wnt signaling (11,12).

Wnts are a family of secreted glycoproteins that bind to transmembrane Frizzled receptors and initiate signaling cascades, and it has been previously noted that they play indispensable roles during cell proliferation, migration, adhesion and survival (13-15). Activation of the Wnt/ $\beta$ -catenin signaling pathway leads to  $\beta$ -catenin nuclear translocation and complex formation with lymphoid enhancer-binding factor/T cell factor (LEF/TCF) transcription factors, followed by transcriptional activation of target genes in the nucleus (13-15). In previous studies using animal models of intimal hyperplasia, increased  $\beta$ -catenin levels have been noted, and the role of Wnt signaling in VSMCs has thus been considered (16,17). Wnt signaling is a novel regulator of VSMC proliferation and, thereby, intimal hyperplasia (17). These findings suggest that kindlin-2 and Wnts have overlapping functions in regulating cell behavior and physiological processes. However, kindlin-2-mediated

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interactions are not yet fully understood, and the relationship between kindlin-2 and Wnt signaling remains to be fully elucidated. Moreover, little is known about the role kindlin-2 plays in VSMC proliferation, migration and intimal hyperplasia.

In the present study, we used RNA interference (RNAi) to examine the effects of kindlin-2 on recombinant Wnt3a-induced VSMC proliferation and migration *in vitro* and intimal hyperplasia following balloon catheter injury of the carotid artery of rats *in vivo*.

## Materials and methods

**Construction and production of lentiviral vectors.** Three siRNA sequences targeting rat kindlin-2 (GenBank accession no., NM\_001011915) and a negative control sequence were constructed by Genechem (Shanghai, China). The kindlin-2 siRNA sequence which performed best was CAAACA GATAACAGCACGG, and that of negative control siRNA (NC siRNA) was TTCTCCGAACGTGTCACGT (data not shown). Short hairpin RNAs (shRNAs) were inserted into the lentiviral vector GV118 driven by the U6 promoter and containing the green fluorescent protein (GFP) reporter gene. All constructs were then verified by sequence analysis. Lentivirus-encoded shRNA against kindlin-2 and the control were produced by co-transfecting 293T cells (purchased from GeneChem, Shanghai, China) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to standard protocols. The virus titers, measured in 293T cells as transducing units/milliliter (TU/ml), were approximately  $1 \times 10^9$  TU/ml.

**Rat model of carotid artery injury and lentiviral transfection.** All animal protocols complied strictly with the Institutional Animal Care and Use Committee guidelines. The procedure for balloon injury in rat carotid arteries has been described previously (18). Briefly, male Sprague-Dawley (SD) rats ( $n=45$ ) (Wuhan University Experimental Animal Center, Wuhan, China), 3–4 months old and weighing 350–400 g were anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg). After intravenous injection of 100 U/kg of heparin sodium, the left common, external and internal carotid arteries were exposed, and a balloon angioplasty catheter (balloon diameter 1.25 mm, balloon length 15 mm; Medtronic, Minneapolis, MN, USA) was introduced through the external carotid arteriotomy incision, advanced to the aortic arch, inflated to produce moderate resistance, and gradually withdrawn 3 times. For lentiviral transfection, a 50  $\mu$ l solution of kindlin-2 siRNA-GFP lentivirus ( $2 \times 10^8$  TU/ml) or NC siRNA-GFP lentivirus ( $2 \times 10^8$  TU/ml) was infused into the injured common carotid artery segment (approximately 20 mm in length) isolated by two microvascular clips, and incubated for 30 min. The external carotid artery was then ligated, and blood flow through the common and internal carotid arteries was restored.

**Hematoxylin and eosin (H&E) staining.** The rats were sacrificed by jugular vein blood collection, which leads to massive loss of blood and death in rats, 4 weeks after balloon injury and lentiviral transfection, and the left common carotid arteries were harvested, fixed in 4% paraformaldehyde, and then embedded in paraffin. For harvesting the arteries, the rats were fixed on a board after being anesthetized. The left common

carotid arteries were exposed by layer separation after skin incision, and the left common carotid arteries were then clipped after clamping the proximal and distal ends of the arteries. For morphologic analysis of intimal hyperplasia, five round cross-sections (4  $\mu$ m thickness) were cut from the approximate middle of the artery, stained with H&E, photographed, and analyzed using Image-Pro Plus 6.0 professional image analysis software (Media Cybernetics, Silver Spring, MD, USA). The intimal and medial cross-sectional areas of the carotid arteries were measured, and the intima/media ratios were also calculated.

**VSMC culture and transfection.** Primary VSMCs were isolated from the thoracic aortas of male SD rats (100–150 g,  $n=16$ ). The rats were fixed on a board after being anesthetized. The thoracic arteries were exposed and clipped. The thoracic arteries were then placed in a dish with Dulbecco's modified Eagle's medium (DMEM) and the endothelial cells were removed by ophthalmic tweezers. Finally, the remaining cells were the VSMCs. The cells were then cultured in DMEM containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. The purity of the VSMCs was assessed at approximately 90% through studying the cell morphology and immunostaining with anti- $\alpha$ -actin antibody (A5228; Sigma, St. Louis, MO, USA). All VSMCs were used for experiments between the 3rd and 6th passages. VSMCs ( $1 \times 10^5$ ) were plated in 6-well plates and grown to approximately 50% confluence, then transfected with kindlin-2 siRNA lentivirus or NC siRNA lentivirus at multiplicities of transfection (MOI) of 100. Lentiviral transfection was validated by visualization of enhanced GFP under a fluorescence microscope (Nikon TE2000; Nikon, Tokyo, Japan).

**Cell proliferation assay.** Cell proliferation was measured by studying the incorporation of bromodeoxyuridine (BrdU) during DNA synthesis in proliferating cells. Untreated VSMCs were seeded at a density of 5,000 cells/well in 96-well culture plates in DMEM with 10% FBS and cultured for 24 h. VSMCs were then starved in DMEM without FBS for 12 h to achieve synchronous growth, and transfected with kindlin-2 siRNA lentivirus or NC siRNA lentivirus at MOI of 100 for 12 h. Wnt3a (final concentration, 100 ng/ml; R&D Systems, Minneapolis, MN, USA) was added to each well after transfection, and 48 h after transfection 20  $\mu$ l BrdU was also added to each well to label the cells during 24 h of incubation. Quantification of BrdU incorporation was performed using a BrdU cell proliferation assay kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. The absorbance was read at a wavelength of 450 nm with a spectrophotometric plate reader (Infinite M200 PRO; Tecan, Männedorf, Switzerland).

**Cell migration assay.** The VSMC migration assay was performed using Transwell cell culture inserts (Corning, High Wycombe, UK) in 24-well plates. One hundred microliters of VSMCs, which were stably transfected with kindlin-2 siRNA, NC siRNA or left untreated ( $3 \times 10^5$  cells/ml), suspended in serum-free DMEM, were added to the upper polycarbonate membrane insert (pore size, 8  $\mu$ m). Wnt3a (final concentration, 100 ng/ml) was added to the upper chamber, and 600  $\mu$ l 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 an atmosphere with 5% CO<sub>2</sub> 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 was used to determine the number of migratory cells by counting the cells in 5 randomly selected fields of view. All experiments were performed in triplicate.

**Reverse transcription-quantitative PCR (RT-qPCR).** After transfection and Wnt3a (100 ng/ml) stimulation for 3 days, total RNA was isolated from VSMCs using TRIzol (Invitrogen) reagent. For RT-qPCR, we performed reverse transcription to produce cDNA from total RNA with oligo(dT), and the fragments were then amplified with a SYBR-Green-based assay kit (Invitrogen) according to the manufacturer's instructions. Thermal cycling conditions comprised an initial denaturation step at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles (95°C for 30 sec; 60°C for 30 sec).  $\beta$ -actin was used for normalization, and the data were analyzed using the 2<sup>- $\Delta\Delta C_t$</sup>  method. The primers were as follows: kindlin-2 forward, 5'-AGATCTGGCTTCGCTGTGAT-3' and reverse, 5'-CGGGATTGATGTCAGTTGTG-3'; c-myc forward, 5'-CGAGCTGAAGCGTAGCTTTT-3' and reverse, 5'-CTCGCCGTTTCCTCAGTAAG-3'; cyclin D1 forward, 5'-GCGTACCCTGACACCAATCT-3' and reverse, 5'-GGCTCCAGAGACAAGAAACG-3';  $\beta$ -actin forward, 5'-CACGATGGAGGGGCGGACTCATC-3' and reverse, 5'-TAAAGACCTCTATGCCAACACAGT-3'.

**Western blot analysis.** After transfection, VSMCs treated with Wnt3a (100 ng/ml) and PBS (control) for 3 days were harvested. The cells were lysed with RIPA buffer supplemented with proteinase inhibitors for 30 min on ice. Protein concentration was measured with a BCA protein assay (Pierce, Rockford, IL, USA). Proteins were then separated by SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 10% non-fat dry milk, and then immunoblotted overnight at 4°C with antibodies that recognize kindlin-2 (1:500; K3269; Sigma),  $\beta$ 1-integrin (1:500; 04-1109; Millipore), total  $\beta$ -catenin (1:1,000; 9562), phospho- $\beta$ -catenin (Ser675, 1:1,000; 9567) (both from Cell Signaling Technology, Beverly, MA, USA), total glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ , 1:400; Sc-9166), phospho-GSK-3 $\beta$  (Ser9, 1:500; Sc-373800) and  $\beta$ -actin (1:1,000; Sc-32251) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After three washes, the blots were incubated with peroxidase-conjugated secondary antibodies (Pierce) for 1 h at room temperature, and subsequently analyzed by an ECL detection system (Beijing Liuyi Instrument Factory, Beijing, China).

**Flow cytometric analysis.** VSMCs were transfected with lentiviruses of kindlin-2 siRNA or NC siRNA and supplemented with Wnt3a (100 ng/ml) for 3 days.  $\beta$ 1-integrin expression on the VSMC surface was evaluated by indirect immunofluorescence using flow cytometry. After being rinsed in PBS, the cells were incubated with rabbit anti-rat antibody against  $\beta$ 1-integrin (1:70) and mouse anti-rat antibody against active  $\beta$ 1-integrin (1:200; MAB2079Z) (both from Millipore) for 30 min at room temperature in the dark. The cells were then washed again and

incubated with phycoerythrin (PE)-conjugated goat anti-rabbit secondary IgG (1:50; bs-0295G) and Cy3-conjugated goat anti-mouse secondary IgG (1:50; bs-0296G) (both from Bioss, Beijing, China) for 45 min and analyzed by flow cytometry using Becton-Dickinson FACSCalibur and CellQuest software.

**Statistical analysis.** All statistical analysis was performed with Statistical Product and Service Solutions 17.0 software (SPSS 17.0). Data are presented as the means  $\pm$  SEM. All values were analyzed using the Student's t-test for comparisons between two groups or one-way ANOVA for multiple comparisons. A P-value <0.05 was considered to indicate a statistically significant difference.

## Results

**Kindlin-2 RNAi suppresses intimal hyperplasia.** Four weeks after balloon injury and lentiviral transfection, the degree of intimal hyperplasia was evaluated morphologically and quantitatively (Fig. 1). Kindlin-2 siRNA lentivirus treatment significantly reduced intimal hyperplasia (P<0.05), and the intima/media ratio was also markedly lower in kindlin-2 siRNA lentivirus-transfected arteries (0.687 $\pm$ 0.117) than in NC siRNA lentivirus-transfected arteries (1.545 $\pm$ 0.277) (P<0.05).

**Kindlin-2 RNAi attenuates the VSMC proliferation induced by Wnt3a.** As shown in Fig. 2C, kindlin-2 mRNA expression was dramatically reduced in VSMCs transfected with kindlin-2 siRNA lentivirus (P<0.05), but not in VSMCs transfected with NC siRNA lentivirus (P>0.05). Compared with the control group, a 47% reduction of kindlin-2 mRNA was observed in VSMCs transfected with kindlin-2 siRNA lentivirus (P<0.05), indicating that kindlin-2 RNAi is effective. Subsequently, we examined the effect of kindlin-2 RNAi on Wnt3a-induced VSMC proliferation by measuring the nuclear incorporation of BrdU (DNA synthesis) and the mRNA expression levels of c-myc and cyclin D1, which are critical genes involved in cell cycle progression and cell proliferation; Wnt3a is a prominent member of the Wnt family and can activate the canonical Wnt pathway and induce cell proliferation (19,20). We observed that kindlin-2 RNAi resulted in significant inhibition of BrdU incorporation compared with the control group and the NC siRNA group with or without Wnt3a stimulation (P<0.05; Fig. 2A). The c-myc and cyclin D1 mRNA expression levels were significantly suppressed by kindlin-2 siRNA lentivirus at MOI of 100 (P<0.05; Fig. 2B and D). NC siRNA lentivirus, which encodes for a non-homologous shRNA, did not affect c-myc and cyclin D1 mRNA expression. After exposure of VSMCs to Wnt3a at a concentration of 100 ng/ml for 3 days, the levels of c-myc and cyclin D1 mRNA were significantly upregulated by 1.9- and 2.3-fold, respectively (P<0.05). By contrast, Wnt3a-induced expression of c-myc and cyclin D1 was also markedly inhibited by pretreatment with kindlin-2 siRNA lentivirus (P<0.05).

**Kindlin-2 regulates cell proliferation by Wnt/ $\beta$ -catenin signaling.**  $\beta$ -catenin and GSK-3 $\beta$  were examined in this study, as they are considered to be the major downstream Wnt signaling molecules (13-15). Kindlin-2 has been found to be coexpressed with  $\beta$ -catenin in the invasive front of tumors and in tumor cells (11). To understand the mechanisms

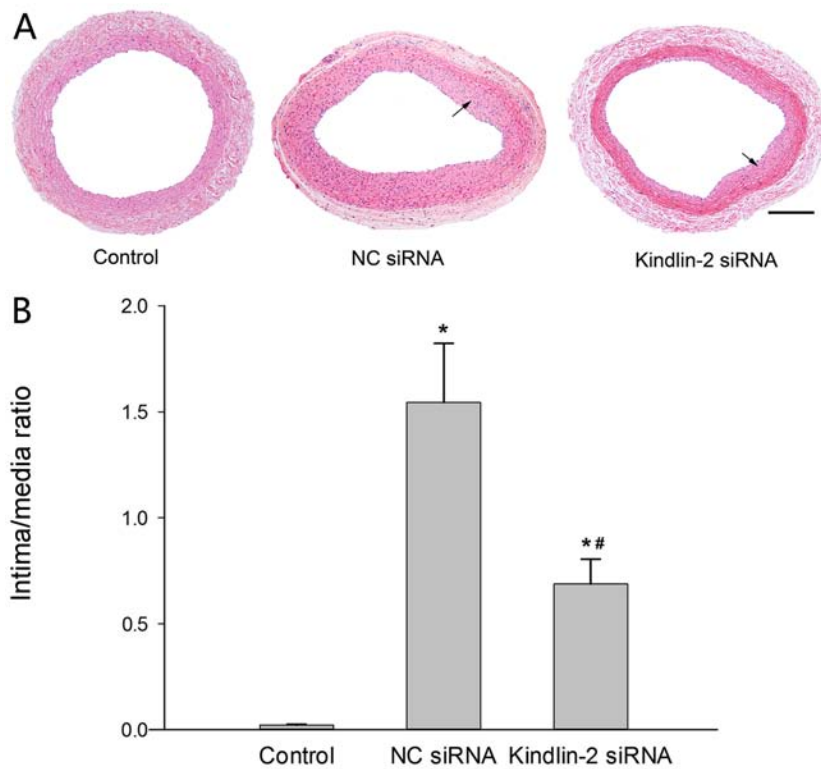


Figure 1. Effects of kindlin-2 silencing on intimal formation in balloon-injured carotid arteries. (A) Representative histologic sections of hematoxylin and eosin-stained carotid arteries. Scale bar represents 100  $\mu$ m. (B) Quantitative analysis of ratio of intima to media was calculated 4 weeks after balloon injury and lentiviral transfection. \* $P < 0.05$  vs. control group; # $P < 0.05$  vs. negative control (NC) siRNA group.

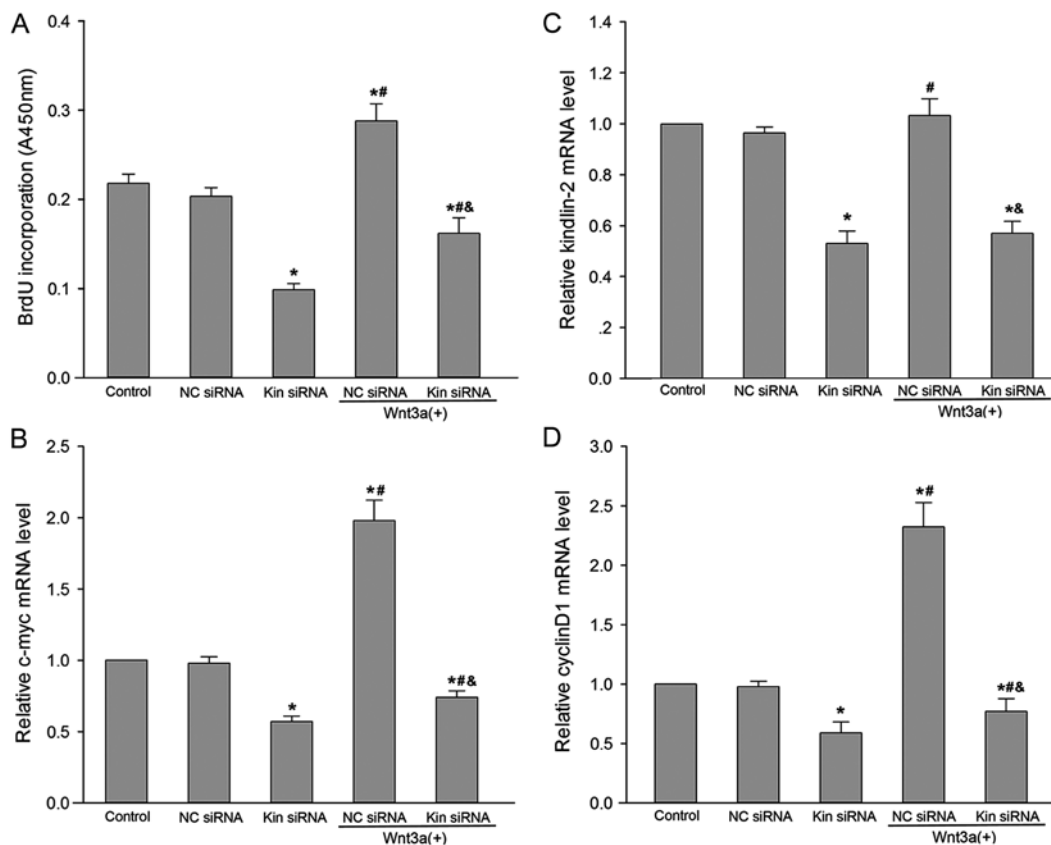


Figure 2. Inhibitory effect of kindlin-2 RNAi on Wnt3a-induced vascular smooth muscle cell (VSMC) proliferation. VSMCs were stimulated for 3 days with recombinant Wnt3a (100 ng/ml) after lentiviral transfection. Cell proliferation was evaluated by (A) BrdU incorporation assay and (B) studying the c-myc, (C) kindlin-2 and (D) cyclin D1 mRNA expression levels. Kindlin-2 siRNA (Kin siRNA) transfection of VSMCs resulted in a significant inhibition of BrdU incorporation. RT-qPCR indicated the efficient suppression of kindlin-2, c-myc and cyclin D1 mRNA in Wnt3a- and kindlin-2 siRNA-treated VSMCs. \* $P < 0.05$  vs. control group and negative control (NC) siRNA group; # $P < 0.05$  vs. kindlin-2 siRNA group; & $P < 0.05$  vs. NC siRNA + Wnt3a group.

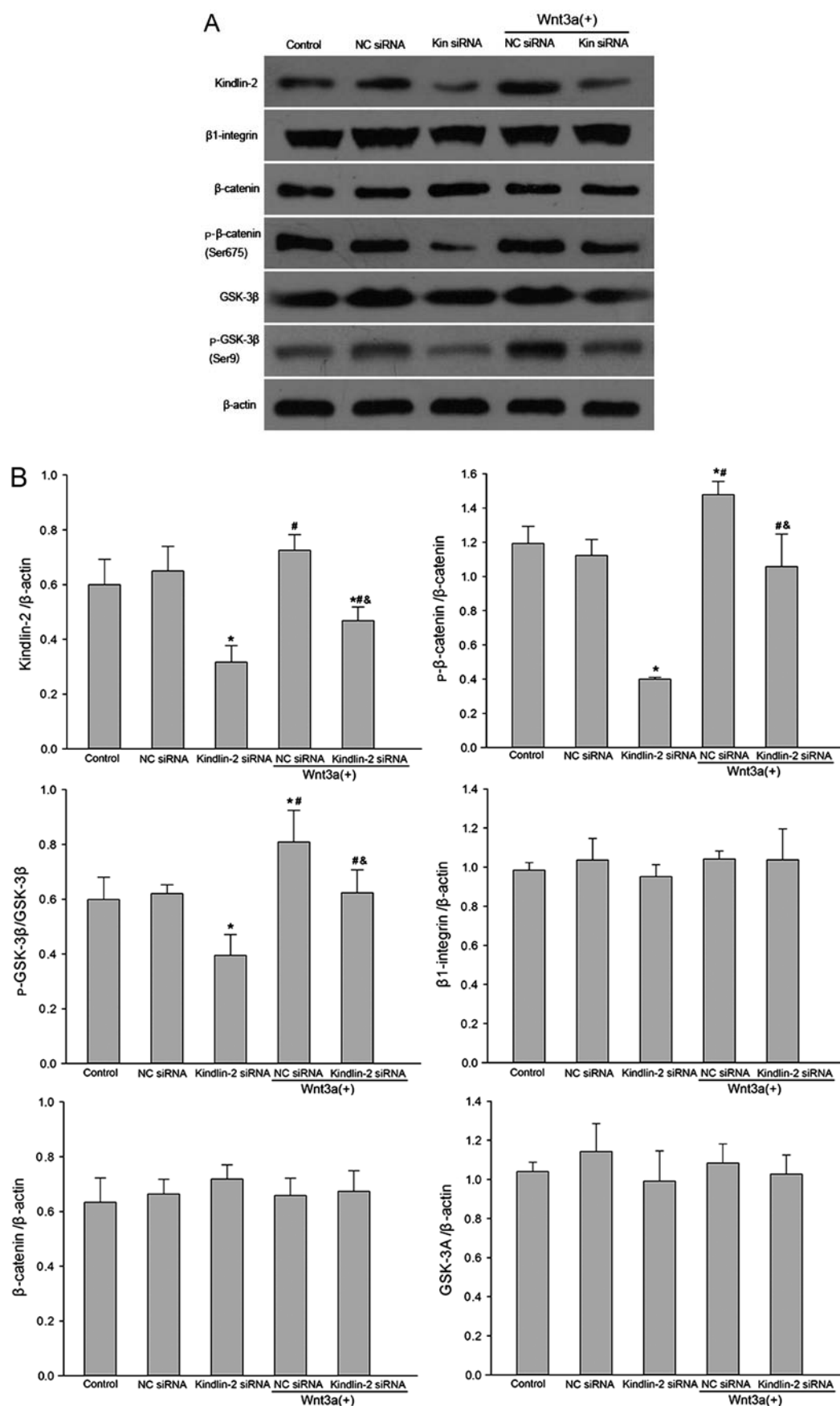


Figure 3. Kindlin-2 knockdown suppresses cell proliferation via Wnt/ $\beta$ -catenin signaling. Vascular smooth muscle cells (VSMCs) were treated with Wnt3a (100 ng/ml) for 3 days after lentiviral transfection. (A) Protein expression of kindlin-2,  $\beta$ 1-integrin, total  $\beta$ -catenin, p- $\beta$ -catenin (Ser675), total glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) and p-GSK-3 $\beta$  (Ser9) were examined by western blot analysis. (B) The kindlin-2,  $\beta$ 1-integrin, total  $\beta$ -catenin and total GSK-3 $\beta$  protein signals were quantified and normalized to that of  $\beta$ -actin. The p- $\beta$ -catenin and p-GSK-3 $\beta$  protein signals were normalized to those of total  $\beta$ -catenin and total GSK-3 $\beta$ , respectively. \* $P < 0.05$  vs. control group and negative control (NC) siRNA group; # $P < 0.05$  vs. kindlin-2 siRNA group; & $P < 0.05$  vs. NC siRNA + Wnt3a group.

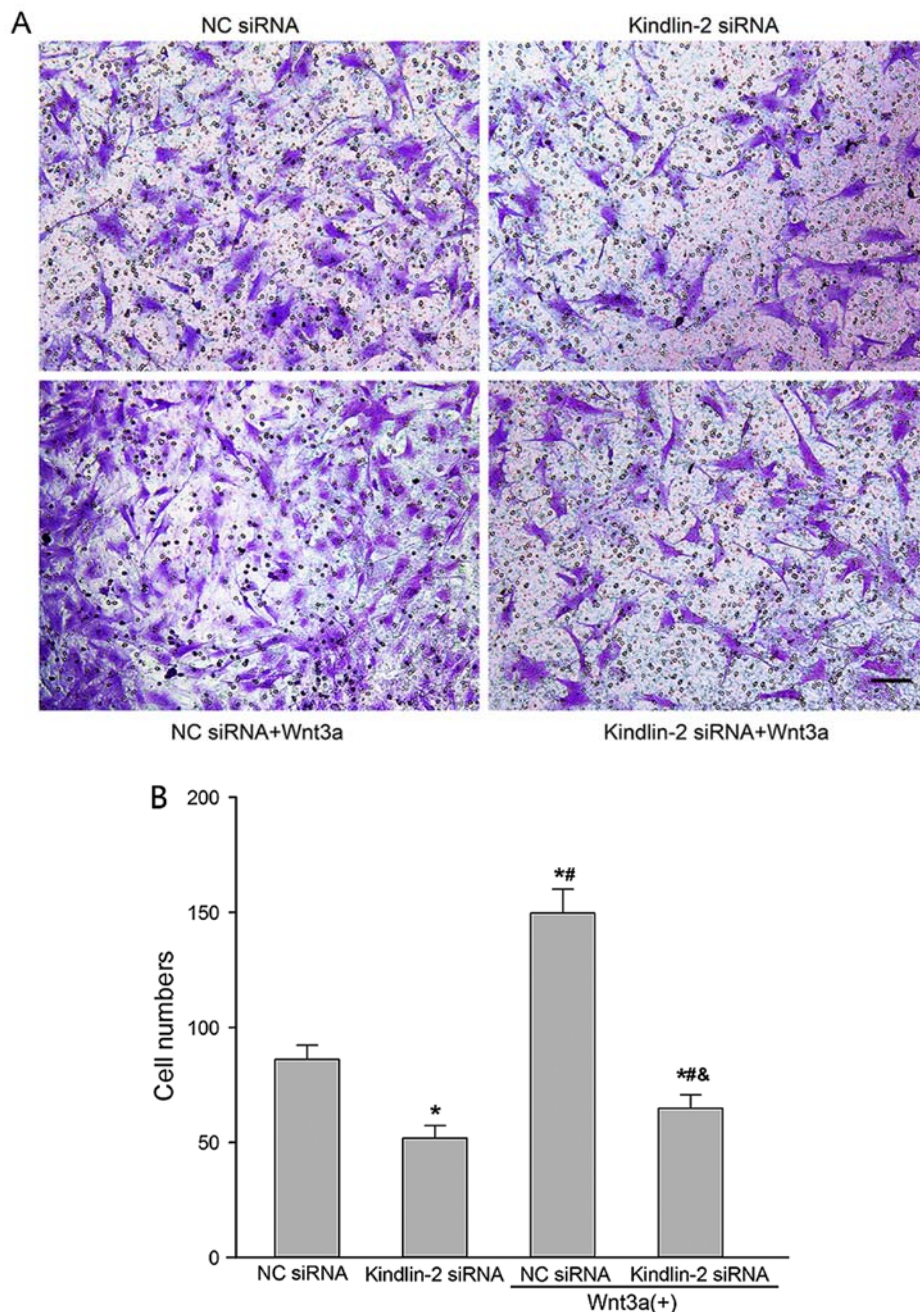


Figure 4. Kindlin-2 RNAi impairs vascular smooth muscle cell (VSMC) migration. (A) VSMCs on Transwell inserts were stained with 0.1% crystal violet (x200). Scale bar, 100  $\mu$ m. (B) The number of migratory VSMCs transfected with kindlin-2 siRNA lentivirus was significantly lower than that of negative control (NC) siRNA group and Wnt3a treatment group. \* $P < 0.05$  vs. NC siRNA group; \*\* $P < 0.05$  vs. kindlin-2 siRNA group; \*#& $P < 0.05$  vs. NC siRNA + Wnt3a group.

of kindlin-2 knockdown on VSMC proliferation induced by Wnt3a, we analyzed the protein expression of kindlin-2,  $\beta$ 1-integrin,  $\beta$ -catenin and GSK-3 $\beta$  (Fig. 3). Our results showed that kindlin-2 knockdown significantly decreased protein expression levels of kindlin-2, p- $\beta$ -catenin (Ser675) and p-GSK-3 $\beta$  (Ser9) ( $P < 0.05$ ). Treatment of VSMCs with Wnt3a upregulated the expression of kindlin-2, p- $\beta$ -catenin (Ser675) and p-GSK-3 $\beta$  (Ser9) ( $P < 0.05$ ). However, the expression of  $\beta$ 1-integrin, total  $\beta$ -catenin and total GSK-3 $\beta$  did not differ significantly between groups ( $P > 0.05$ ).

**Kindlin-2 depletion impairs VSMC migration.** We performed a Transwell migration assay to investigate the effect of

kindlin-2 RNAi and Wnt3a treatment on VSMC migration. The migratory ability of VSMCs transfected with kindlin-2 siRNA lentivirus was significantly decreased ( $P < 0.05$ ; Fig. 4). After treatment with Wnt3a, the migratory ability of VSMCs was significantly increased ( $P < 0.05$ ). The number of cells that migrated across the polycarbonate membrane was higher in the NC siRNA group and Wnt3a treatment group than in the kindlin-2 siRNA group ( $P < 0.05$ ).

**Kindlin-2 and Wnt3a regulate integrin activation.** Since kindlin-2 knockdown and treatment with Wnt3a did not markedly affect the protein expression of  $\beta$ 1-integrin, we quantified the expression of active  $\beta$ 1-integrin on the surface of VSMCs



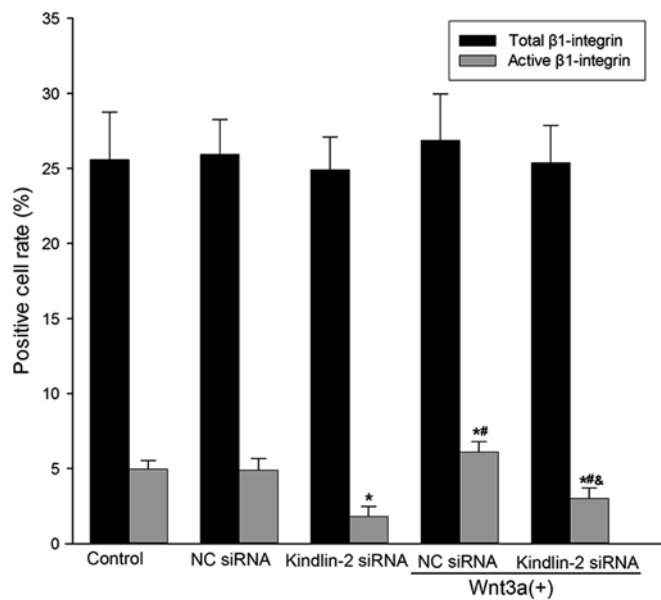


Figure 5. Kindlin-2 and Wnt3a regulate  $\beta 1$ -integrin activation. Active  $\beta 1$ -integrin and total  $\beta 1$ -integrin expression on the surface of vascular smooth muscle cells (VSMCs) was evaluated by flow cytometry. Kindlin-2 knockdown significantly reduced the active- $\beta 1$ -integrin levels on the VSMC surface. Treatment with Wnt3a activated  $\beta 1$ -integrin but did not markedly affect the expression levels on the cell surface. \* $P < 0.05$  vs. control group and negative control (NC) siRNA group; # $P < 0.05$  vs. kindlin-2 siRNA group; & $P < 0.05$  vs. NC siRNA + Wnt3a group.

using flow cytometry. The anti-active- $\beta 1$ -integrin antibody is specific for the active conformation of rat  $\beta 1$ -integrin, and it can also discriminate between the different activated states. Therefore, it is exceptionally useful for studying how  $\beta 1$ -integrin activation is regulated. Our results showed that knockdown of kindlin-2 significantly reduced the VSMC surface levels of active- $\beta 1$ -integrin ( $P < 0.05$ ; Fig. 5). VSMCs that were stimulated with Wnt3a for 3 days after transfection bound to more active- $\beta 1$ -integrin antibody than the kindlin-2 siRNA group ( $P < 0.05$ ). However, kindlin-2 knockdown or Wnt3a treatment did not have a marked effect on the total amount of  $\beta 1$ -integrin expression on the surface of VSMCs ( $P > 0.05$ ; Fig. 5). These results demonstrate that a minimal level of kindlin-2 is required for optimal integrin activation in VSMCs, and that Wnt3a treatment activates  $\beta 1$ -integrin without changing its expression levels.

## Discussion

In the present study, to the best of our knowledge, we have provided the first direct evidence that kindlin-2 regulates VSMC proliferation and migration *in vitro* and intimal hyperplasia *in vivo*. Additionally, we have shown that Wnt/ $\beta$ -catenin signaling is involved in signal transduction and the functional regulation of kindlin-2. Consequently, we propose that modification of kindlin-2 or Wnt signaling is a potential target for inhibition of VSMC proliferation, migration and intimal hyperplasia.

Kindlin-2 belongs to the kindlin family of proteins; the kindlins are emerging as a novel class of molecules which are implicated in integrin activation, a critical process for cell proliferation, migration, differentiation and adhesion as well as for cell-ECM interactions (7). They comprise three

evolutionarily conserved members in vertebrates, kindlin-1, kindlin-2 and kindlin-3, which share considerable sequential and structural similarities (7,21-24). The kindlins have a FERM domain, which is interrupted by a pleckstrin homology domain, and bind directly to various classes of integrins as well as participating in inside-out integrin activation (7,21-25). Loss-of-function mutations in kindlin-1 and kindlin-3 cause Kindler syndrome and leukocyte adhesion deficiency III syndrome, respectively, but no human disease has yet been associated with the kindlin-2 gene (7,22-24).

At present, limited information exists on the physiological functions of kindlin-2, and the data are mainly derived from knockout animal models and *in vitro* studies with cell lines. The essential role of kindlin-2 in development is demonstrated by its peri-implantation lethality and the abnormal heart development noted in kindlin-2 knockout mice and zebrafish (8,9). At the cellular level, kindlin-2<sup>-/-</sup> embryonic stem cells exhibited a normal proliferation rate, but strongly reduced adhesion to laminins and fibronectin was noted (8). In C2C12 cells, kindlin-2 contributed to myocyte elongation and fusion in multinucleated myotubes (26). Knockdown of kindlin-2 in wild-type keratinocytes impaired cell spreading (27). Kindlin-2 is also important in the regulation of podocyte-matrix adhesion and matrix deposition (28). Our results revealed that kindlin-2 regulates VSMC proliferation and migration. However, in previous studies, overexpression and knockdown of kindlin-2 have yielded contradicting results regarding specific functions: evidence for both suppression of cancer cell invasion in leiomyosarcoma or colon carcinoma cell lines (3,29) and stimulation of cell migration in fibroblasts, human umbilical vein endothelial cells (HUVECs), malignant mesothelioma and gastric cancer cells has been noted (6,30-32). These observations suggest that the functions of kindlin-2 are cell type- or integrin-specific, and that its role in cell motility differs depending on the biological context. For example, overexpression of kindlin-2 in Chinese hamster ovary (CHO) cells exogenously expressing  $\alpha IIb\beta 3$  integrin enhances its activation (3,30). However, overexpression of kindlin-2 in the same type of cells inhibits endogenous  $\alpha 5\beta 1$ -integrin activation (21). Taken together, these results suggest that alterations of kindlin-2 expression affect integrin-dependent functions. This study, and others, have observed that kindlin-2 knockdown did not markedly affect the protein expression of  $\beta 1$ -integrin, but significantly reduced  $\beta 1$ -integrin activation (6,28).

To date, kindlin-2 is the only kindlin protein that has not been implicated in disease pathophysiology, but it is rapidly emerging as a key molecule in cardiac and muscle development (7). Moreover, the role of kindlin-2 in cell proliferation and migration is not yet fully understood, and little is known about the signal transduction pathways of kindlin-2; determining these will be important in understanding the role of kindlin-2 in the pathophysiology of disorders of integrin activation. To determine the effect of kindlin-2 on cell proliferation and migration, in the present study, RNA-mediated interference experiments were performed on VSMCs. siRNAs targeting kindlin-2 or irrelevant RNA as a negative control were constructed and introduced to VSMCs, and kindlin-2 expression levels were analyzed by RT-qPCR and western blot analysis. Transfection with kindlin-2 siRNA but not NC siRNA significantly inhibited the expression of kindlin-2. We detected

VSMC proliferation by measuring the nuclear incorporation of BrdU and VSMC migration using a Transwell assay. The results showed that kindlin-2 siRNA resulted in significant inhibition of BrdU incorporation compared with the NC siRNA group, and the migratory ability of VSMCs in the kindlin-2 siRNA group was significantly decreased.

Although these previous studies have suggested that kindlin-2 signaling is crucial to VSMC proliferation and migration, the underlying mechanism is still unknown. Previous studies have shown that kindlin-2 is important to the enhancement of Wnt/ $\beta$ -catenin signaling, as it selectively binds to active  $\beta$ -catenin and stabilizes it by preventing GSK-3 $\beta$  (a negative regulator of Wnt signaling) from binding (11,12). It is well known that the major downstream Wnt signaling target is  $\beta$ -catenin. Activation of the  $\beta$ -catenin signaling pathway occurs by inhibiting GSK-3 $\beta$  from phosphorylating the N-terminal part of  $\beta$ -catenin, which leads to rapid degradation of  $\beta$ -catenin. However,  $\beta$ -catenin is also activated by phosphorylation in the C-terminal at serine 675, which facilitates the translocation of  $\beta$ -catenin to the nucleus and enhances its transcriptional activity (20). In this study, we activated Wnt/ $\beta$ -catenin signaling with recombinant Wnt3a to study the effect of kindlin-2 siRNA on VSMCs. We noted increased  $\beta$ -catenin phosphorylation at Ser675 and increased GSK-3 $\beta$  phosphorylation at Ser9 (inactivation of GSK-3 $\beta$ ) after Wnt3a stimulation. Kindlin-2 knockdown significantly decreased protein expression levels of p- $\beta$ -catenin (Ser675) and p-GSK-3 $\beta$  (Ser9). However, the expression of total  $\beta$ -catenin and GSK-3 $\beta$  was not markedly affected by kindlin-2 siRNA. Moreover, we noted that Wnt3a also elevated  $\beta$ 1-integrin activity and promoted VSMC migration.

Previous studies have revealed that Wnt/ $\beta$ -catenin signaling is involved in the regulation of VSMC proliferation and migration (14-17,33). Wang *et al* showed that overexpression of constitutively active  $\beta$ -catenin increased cyclin D1 promoter activity in a rat VSMC line (34). Cyclin D1 protein is important for the regulation of the cell proliferation cycle. When cyclin D1 is amplified or expressed, the G1/S transition is shortened and cell proliferation is promoted; c-myc, which was investigated in the present study, is known to act as a proto-oncogene and cell proliferation-initiating factor (35). It has previously been pointed out that c-myc and cyclin D1 are downstream target genes of the Wnt/ $\beta$ -catenin signaling pathway (20,36). In the present study, we confirmed that treatment with Wnt3a resulted in the activation of Wnt/ $\beta$ -catenin signaling and increased the expression of  $\beta$ -catenin responsive genes c-myc and cyclin D1. Taken together, these results show that growth inhibition by kindlin-2 siRNA in VSMCs was manifested partly through the dysregulated nuclear translocation of  $\beta$ -catenin and the consequent downregulation of its transcriptional targets c-myc and cyclin D1.

VSMC proliferation and migration play a critical role in intimal hyperplasia through cellular expansion and ECM deposition (1). Elucidating the molecular mechanisms responsible for VSMC proliferation and migration has led to the development of novel therapeutic approaches. In this study, we have investigated how kindlin-2 silencing inhibits intimal hyperplasia. When we studied the vascular morphology in our study, we noted that intimal hyperplasia was clearly visible 4 weeks after balloon injury and that kindlin-2 siRNA lentivirus treat-

ment significantly reduced intimal hyperplasia. Moreover, the intima/media ratio was also markedly reduced in arteries transfected with kindlin-2 siRNA lentivirus compared with arteries transfected with NC siRNA lentivirus. The possible reason for this result is that kindlin-2 RNAi inhibits VSMC proliferation and migration to the intima. Our data indicate that kindlin-2 knockdown plays dual roles in the treatment and prevention of intimal hyperplasia. Kindlin-2 siRNA not only inhibits VSMC proliferation and migration by Wnt signaling, but also suppresses VSMC migration by regulating  $\beta$ -integrin activation. Our results suggest that inhibition of kindlin-2 is an attractive therapeutic approach for prevention of intimal hyperplasia.

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