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ORIGINAL PAPER

Cdc42 Promotes Schwann Cell Proliferation and Migration Through Wnt/β-Catenin and p38 MAPK Signaling Pathway After Sciatic Nerve Injury

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Abstract Schwann cells (SCs) are unique glial cells in the peripheral nerve and may secrete multiple neurotrophic factors, adhesion molecules, extracellular matrix molecules to form the microenvironment of peripheral nerve regeneration, guiding and supporting nerve proliferation and migration. Cdc42 plays an important regulatory role in dynamic changes of the cytoskeleton. However, there is a little study referred to regulation and mechanism of Cdc42 on glial cells after peripheral nerve injury. The present study investigated the role of Cdc42 in the proliferation and migration of SCs after sciatic nerve injury. Cdc42 expression was tested, showing that the mRNA and protein expression levels of Cdc42 were significantly up-regulated after sciatic nerve injury. Then, we isolated and purified SCs from injuried sciatic nerve at day 7. The purified SCs were transfected with Cdc42 siRNA and pcDNA3.1-Cdc42, and the cell proliferation, cell cycle and migration were assessed. The results implied that Cdc42 siRNA remarkably inhibited Schwann cell proliferation and migration, and resulted in S phase arrest. While pcDNA3.1-Cdc42 showed a contrary effect. Besides, we also observed that Cdc42 siRNA down-regulated the protein expression of β-catenin, Cyclin D1, c-myc and p-p38, which were up-regulated by pcDNA3.1-Cdc42. Meanwhile, the inhibitor of Wnt/β-catenin and p38 MAPK signaling pathway IWP-2 and SB203580 significantly inhibited the effect of pcDNA3.1-Cdc42 on cell proliferation and migration. Overall, our data indicate that Cdc42 regulates Schwann cell proliferation and migration through Wnt/β-catenin and p38 MAPK signaling pathway after sciatic nerve injury, which provides further insights into the therapy of the sciatic nerve injury.

Keywords Cdc42 · Schwann cell · Migration · Sciatic nerve injury

Introduction

Peripheral nerve injury (PNI) is a commonly-encountered disease in clinical, which results in a multitude of changes within an organism, including motor dysfunction, pain and associated cognitive and emotional comorbidities, thus exerting a lasting negative impact on patients' quality of life [1, 2]. Schwann cell (SCs) proliferation are critical for repair and regeneration of PNI. SCs accelerate proliferation and migration in 24 h after nerve injury, and they arrange along with the long axis of the nerve fibers to guide extension of regenerative axon to target structure [3]. Therefore, it is particularly crucial for neural injury and repair. However, the mechanisms of regulating SCs proliferation remain largely unclear.

Cell division cycle protein 42 (Cdc42) is a member of Rho-family proteins and may combine guanine tri-nucleotide(GTP), acting as an important molecular switch factor in cell signal transduction system of mammals, and it has an important regulatory role in the dynamic changes of the cytoskeleton [4]. Previous immunohistochemistry studies indicated that Cdc42 was located in the dorsal root ganglion neurons and axons and SCs of the sciatic nerve, co-immunoprecipitation studies demonstrated associations of pak with Rac and Cdc42 in both the dorsal root ganglion and sciatic nerve [5]. These results suggested that Cdc42 may modulate SCs

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biological function in sciatic nerve via one or multiple signaling pathway. Moreover, previous study indicated that nerve injury caused profound, long-lasting up-regulation of Cdc42 mRNA in the spinal cord, oligodendrocyte-like cells and dorsal root ganglia [6]. Therefore, we deduce that alteration of SCs proliferation after PNI may be associated with Cdc42 regulation. However, there are only a limited studies referred to regulation and mechanism of Cdc42 in SCs after PNI.

Therefore, our study aimed to investigate the expression level and regulatory effect of Cdc42 on proliferation and migration of SCs after sciatic nerve injury. Moreover, the potential molecular mechanism of Cdc42 modulates SCs proliferation and migration after injury was also explored.

Materials and Methods

Animal Surgeries and Tissue Preparation

Fifty-six male Sprague–Dawley (SD) rats (8 weeks, 200–260 g) were randomly divided into control group (n = 10), sham group (n = 10) and sciatic nerve injury group (n = 36). Rats were anaesthetized by an intraperitoneal injection of 10% (v/v) chloral grate (400 mg/kg), and ~2 cm of sciatic nerve was exposed by a longitudinal incision and lifted through an incision on the lateral aspect of the mid-thigh of the left hind limb. The greater sciatic foramen was then resected at the tendon level of the obturator internus muscle, and the incision sites were then closed. The proximal stumps of the sciatic nerve (0.5 cm) were harvested at 0, 1, 4, 7, 14 and 28 days after injury. All surgical interventions and postoperative care involving animals were performed in accordance with the Institutional Animal Care guidelines and were approved by the Administration Committee of Experimental Animals, Xi'an Medical University.

mRNA Isolation and Real-Time PCR

Total RNA was isolated using Trizol reagent and Reverse-transcribed complementary DNA was synthesized using the Prime-Script RT reagent Kit (TaKaRa, Dalian, China). RT-qPCR analysis was performed using 1 μl of cDNA template, 50 ng of each gene specific primer, and SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) on a ABI Prism 7500 system. The RT-qPCR protocol was as follows: 95 °C for 20 s; 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 20 s and extension at 72 °C for 30 s. The target gene relative expression levels were normalized to β-actin.

Western Blot Assays

Protein extracts were prepared from normal or injured sciatic nerve at 1, 4, 7, 14, 28 days using RIPA buffer (Beyotime, Nantong, China) which was added 1% fresh phenylmethanesulfonyl fluoride (PMSF. Beyotime, Nantong, China) and 1% phosphatase inhibitor PhosSTOP (Roche, Shanghai, China). 20 μg proteins were separated by SDS–PAGE and electrotransferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was then blocked with 10% non-fat dry milk for 1 h and incubated with the mouse anti-Cdc42 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-β-catenin, rabbit anti-cyclin D1, mouse anti-c-myc, rabbit anti-p38 and rabbit anti-p-p38 antibody (Cell Signaling Technology, Danvers, MA, USA). After incubating with horseradish peroxidase (HRP) -conjugated rabbit anti-mouse or goat anti-rabbit (Amersham, Little Chalfont, UK), the blots were visualized using an enhanced chemiluminescence system (Pierce, Rockford, IL, USA).

Isolation and Primary Culture of SCs

SCs were isolated from sciatic nerves after injury for 7 days in SD rats according to a previous method [7]. Briefly, nerves, which were already removed epineurium, were strained and minced from rats after being anaesthetized. Then, nerve fragments were incubated with 0.1% collagenase A (Sigma, St. Louis, MO, USA) and 0.25% trypsin (Sigma-Aldrich) for 30 min at 37 °C, and then filtered through a 40-μm cell strainer and centrifuged at 400 g for 6 min. After washing the cell pellet with Dulbecco's Modified Eagle's medium (DMEM)/F-12 containing 10% fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, USA), the pellet was resuspended in DMEM-D-valine, supplemented with 10% FBS, 2 mmol/L L-glutamine (PAA, Austria), 1% antibiotics (PAA Laboratories), N2 supplement (Invitrogen), 10 μg/ml bovine pituitary extract (Sigma-Aldrich), 5 μmol/L forskolin (Sigma-Aldrich). This medium is subsequently referred to as "Schwann cell medium". Finally, cell suspension was plated on dishes coated with poly-L-lysine (Sigma-Aldrich) and laminin (Sigma-Aldrich) at 75,000 cells/cm^2 for 24 h.

Flow-Cytometry Analysis

For purification of SCs, we applied the previous study [8]. Briefly, the dishes were washed with 2 ml Ca^{2+}- and Mg^{2+}-free PBS followed by 2 ml 1 mM EGTA (Sigma-Aldrich). The dishes were agitated for 2–4 min to release rounded-up or detaching cells. The suspension of floating cells that consisted of mainly SCs were collected into a centrifuge tube and centrifuged at 1800 g/w for 10 min. After

removal of the supernatant, the pellet was resuspended and plated as described. For identification of SCs purity, the cells were blocked with a blocking buffer that consisted of 10% (v/v) goat serum and 1 mg BSA/ml in PBS for 1 h. Then the fixed cells were incubated with primary antibodies against p75 low affinity NGF receptor (p75LNGFR, 1:500, Abcam) at room temperature for 2 h or overnight at 4 °C. Next cells were washed and the secondary antibody, goat anti-mouse IgG conjugated-fluorescein isothiocyanate (FITC, 1:50), was incubated for 45 min at 37 °C. The harvested cells were analyzed on a flow-cytometer and counted fraction of p75LNGFR positive cells.

Plasmids and siRNA Transfection

Cdc42 siRNA and its negative control (control siRNA) primers sequences were designed by Invitrogen Block-iT RNAi Designer (appendix) and synthesized by Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Cdc42 siRNA_406: 5′- GCU UGU CGG GAC CCA AAU UdTdT-3′(F), 5′- AAU UUG GGU CCC GAC AAG CdTdT-3′(R). Cdc42 control siRNA_406: 5′- GCU UCG GGA CCC AAU GAU UdTdT-3′(F), 5′- AAU CAU UGG GUC CCG AAG CdTdT-3′(R). The plasmid expression vector pcDNA3.1-Cdc42 and non-specific control vector (pcDNA3.1) were purchased from Cell signaling Technology. SCs were planted into a 6-well plate with 1×10^6/well, then Cdc42 siRNA, control siRNA, pcDNA3.1-Cdc42 and pcDNA3.1 were transfected using Lipofectamine 2000 (Invitrogen) according to the protocol of the manufacturer.

Cell Proliferation Assay

The proliferation of SCs was measured using the Cell-LightTM EdU DNA Cell Proliferation Kit (RiboBio Co. Ltd., Guangzhou, China) according to the manufacturer's instructions. Briefly, SCs were suspended in fresh pre-warmed (37 °C) complete medium, counted, and plated onto 0.01% poly-L-lysine-coated 96-well plates. Then 5-ethynyl-2′-deoxyuridine (EdU) was applied to cell culture, and incubated for additional 24 h. After that, the cells were fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 30 min and then Hoechst 33,342 nucleus staining was applied for the cells. The cell proliferation of SCs was analyzed by using images of randomly selected fields obtained on a DMR fluorescence microscope (Leica Microsystems, Bensheim, Germany).

Cell Migration Assay

Migration of SCs was estimated by transwell-based assay using a 6.5 mm transwell chambers with 8 mm pores. The bottom surface of each membrane was coated with 10 mg/ml fibronectin. SCs (10^6 cells/ml) resuspended in DMEM were placed onto the upper chambers of each transwell and incubated at 37 °C in 5% CO_2 and allowed to adhere for 1 h. The lower chambers were added 600 ml of complete medium. 6 h later, the upper surface of each membrane was removed using a cotton swab. Cells on the lower surface of each membrane were stained with 0.1% Crystal Violet for 15 min, imaged, and counted using a DMR inverted microscope (Leica Microsystems, Deerfield, IL, USA). Assays were performed three times using triplicate wells.

Cell Cycle Assay

For cell cycle analysis, cells were digested with trypsin and washed with phosphate buffered saline (PBS) solution. The cell suspensions were centrifuged at 1000 rpm/min and harvested, then cells were fixed overnight and stained with 50 μg/ml propidium iodide (PI) and 100 μg/ml RNase A at 4 °C for 20 min. The fluorescence intensity was detected at 488 nm using a flow cytometer. Cells were sorted using a FACSCalibur flow cytometer (Becton and Dickinson) and analyzed using CellQuest software (Becton and Dickinson).

Statistical Analysis

Data were presented as mean ± SD (n = 6). The statistical significance was determined by Student's t-test for two groups or by one-way ANOVA followed by Bonferroni's test for multiple groups. Statistical significance was calculated with a P value of <0.05.

Results

Cdc42 is Up-Regulated After Sciatic Nerve Injury

Changes of Cdc42 expression in proximal stumps of the injured sciatic nerve were measured using RT-qPCR and western blot. The Cdc42 mRNA expression level in proximal stumps of the injured sciatic nerve was higher than that in control at 7,14 and 28 d (Fig. 1a). Besides, the expression level of Cdc42 protein was also markedly higher in injured sciatic nerve (Fig. 1b). Since Cdc42 expression is the highest on day 7 after sciatic nerve injury, the SCs at this point in time were isolated. The p75 low affinity nerve growth factor receptor (p75LNGFR) is a marker of SCs [11], thus the fraction of p75LNGFR positive cells were measured using flow-cytometer before and after SCs purification. The result revealed that p75LNGFR positive cells accounted for over 99 percent of the total isolated cells from sciatic nerve after SCs purification(Fig. 1c), suggesting that the purified method is feasible.

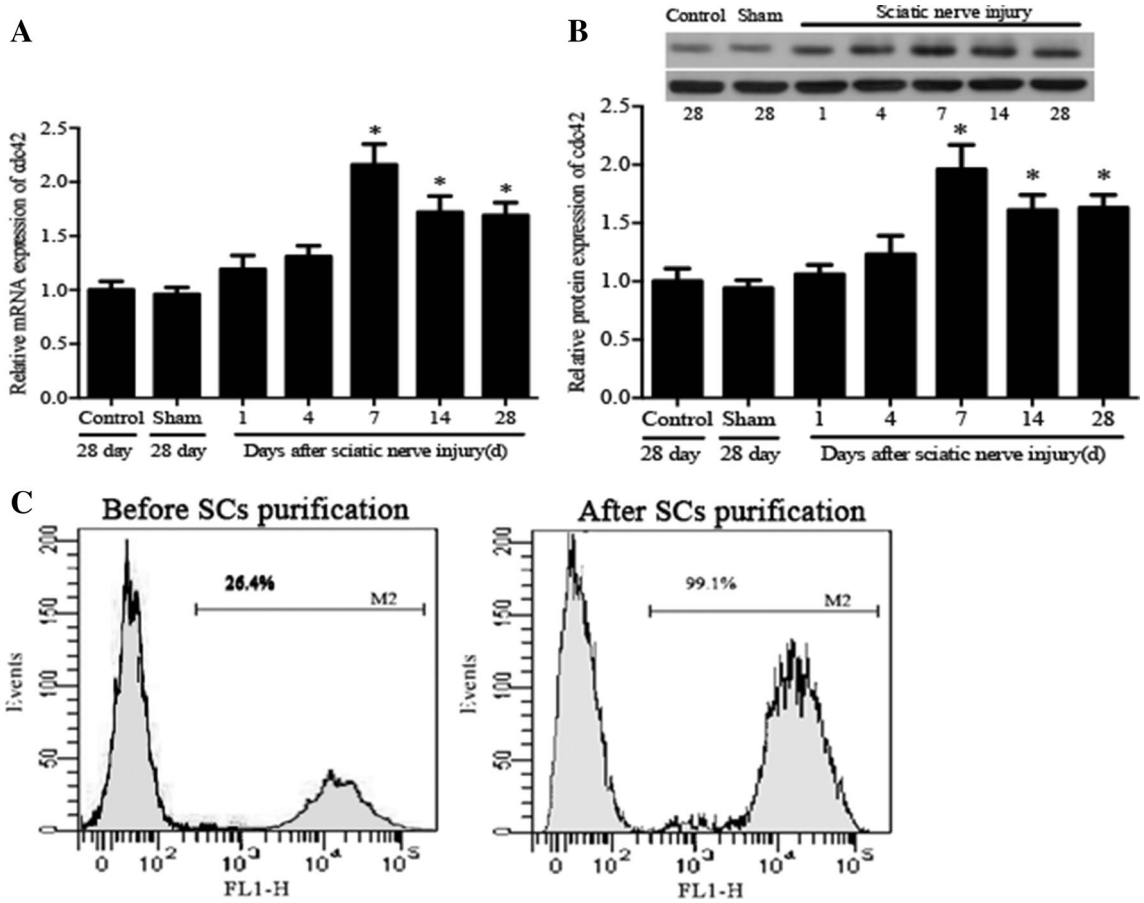

Fig. 1 The expression of Cdc42 in the sciatic nerves after injury. **a** The mRNA expression level of Cdc42 was detected using RT-qPCR assay in proximal stumps of the nerve following sciatic nerve transection at 1, 4, 7, 14, 28 d. **b** Protein expression level of Cdc42 was measured using western blot assay in proximal stumps of the nerve following sciatic nerve transection at 1, 4, 7, 14, 28 d. Results are expressed as the mean ± SD. Each experiment was performed in triplicate. $^{*}P < 0.05$ vs. control. **c** Count by flow-cytometry for p75LNGFR positive cells before and after SCs purification

Cdc42 Regulated Schwann Cell Proliferation and Migration

We first performed proliferation assay in different time points (1, 2, 3, 4, 5, 6, 7 days) to protract cell growth curve (Fig. 2a). Next, SCs were transfected with Cdc42 siRNA, pcDNA3.1-Cdc42 and non-targeting negative control to knockdown or overexpress Cdc42 expression, respectively. The inhibition rate of Cdc42 siRNA was approximately 56%(Fig. 2b). Results showed that transfection of SCs with Cdc42 siRNA significantly inhibited cell proliferation, while Cdc42 overexpressed by pcDNA3.1-Cdc42 remarkably promoted cell proliferation (Fig. 2c). Additionally, Cdc42 silence caused increase of cell content of S phase and reduction of cell content of G2/M phase, while Cdc42 overexpression generated inhibition of cell content of S phase and raise of cell content of G2/M phase in cell cycle (Fig. 2d). These results indicated that alteration of SCs proliferation is connected with change of cell cycle.

Next, we tested the effect of Cdc42 on SCs migration using transwell migration assay. The result showed that Cdc42 siRNA markedly inhibited cell migration, while pcDNA3.1-Cdc42 remarkably promoted cell migration of SCs in the injured sciatic nerve (Fig. 2e).

Wnt/β-Catenin and p38 MAPK Signaling Pathway are Activated by Cdc42

To figure out the potential mechanism of Cdc42 promote SCs proliferation and migration, the Wnt/β-catenin and p38 MAPK signaling pathway were studied in this research. As shown in Fig. 3, the β-catenin protein expression level was significantly up-regulated by pcDNA3.1-Cdc42, while statistically markedly down-regulated by Cdc42 siRNA (Fig. 3a). Furthermore, as the down-stream targets of β-catenin, the cyclin D1 and c-myc protein expression level were also elevated by pcDNA3.1-Cdc42, and decreased by Cdc42 siRNA (Fig. 3b, c). Besides, pcDNA3.1-Cdc42

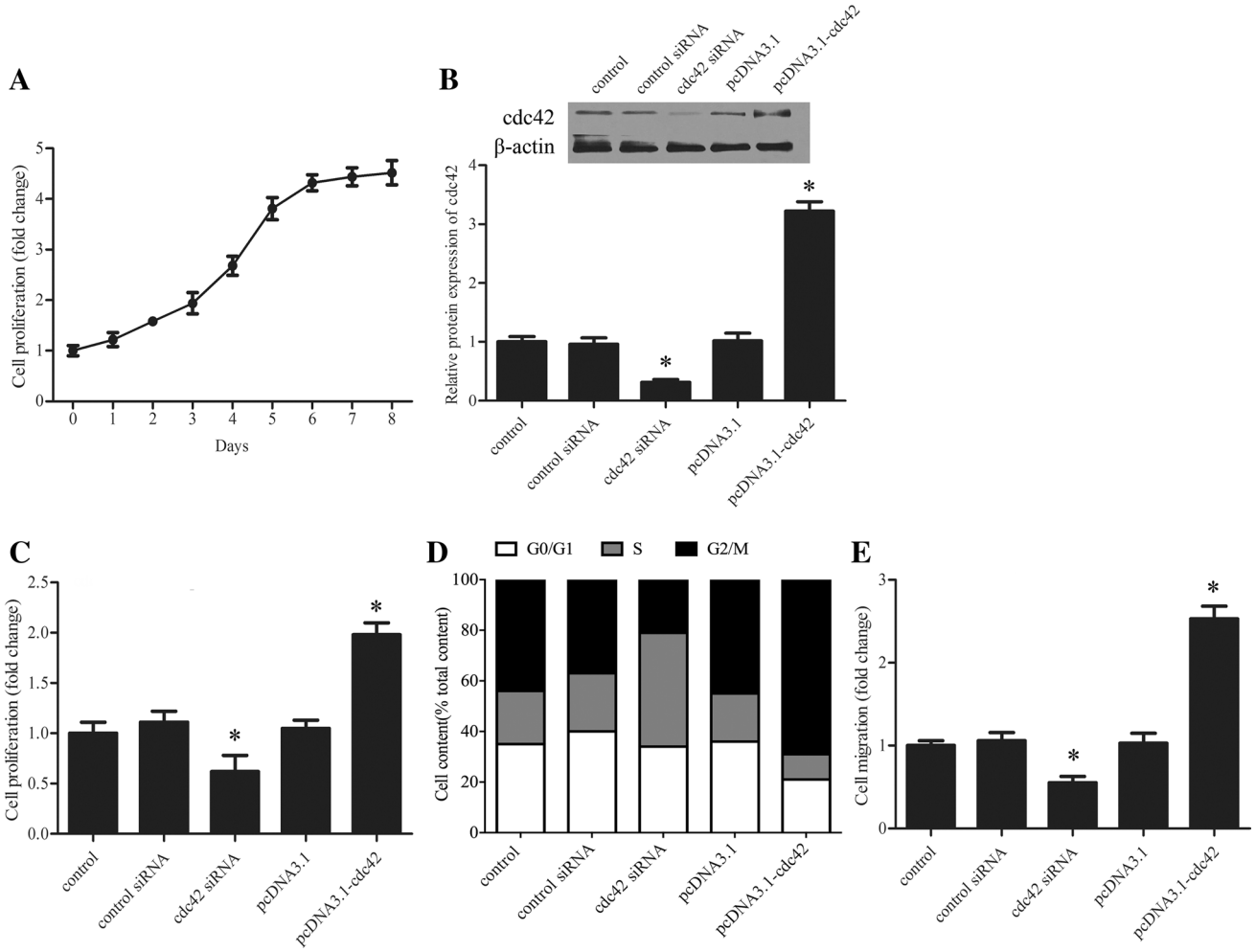

Fig. 2 The Effect of Cdc42 on Schwann cell proliferation and migration in the sciatic nerves after injury. **a** SCs isolated from sciatic nerves after injury for 7 days in SD rats were cultured and cell proliferation were examined in different time points (0, 1, 2, 3, 4, 5, 6, 7 days). **b** SCs cells were transfected with control siRNA, Cdc42 siRNA, pcDNA3.1 and pcDNA3.1-Cdc42, the Cdc42 protein expression level was assessed using western blot assay. **c** SCs cells were transfected with control siRNA, Cdc42 siRNA, pcDNA3.1 and pcDNA3.1-Cdc42, cell proliferation was investigated using Cell-LightTM EdU DNA Cell Proliferation Kit. **d** SCs cells were transfected with control siRNA, Cdc42 siRNA, pcDNA3.1 and pcDNA3.1-Cdc42, cell migration was detected by transwell-based assay. **e** Cell cycle was analyzed by flow cytometry. Results are expressed as the mean ± SD. Each experiment was performed in triplicate. $^*P < 0.05$ vs. control

remarkably increased the arbitrary of p38 (p-p38 /p38), which was decreased by Cdc42 siRNA (Fig. 3d).

Cdc42 Regulates Schwann Cell Proliferation and Migration Through Wnt/β-Catenin and p38 MAPK Signaling Pathway

To explore the role of Wnt/β-catenin and p38 MAPK signaling in the cac42 mediated Schwann cell proliferation and migration in the injured sciatic nerve, SCs were treated with pcDNA3.1-Cdc42, Wnt/β-catenin inhibitor IWP-2 and p38 MAPK inhibitor SB203580, then the cell viability and migration were determined. As a result, pcDNA3.1-Cdc42 significantly increased, while IWP-2 and SB203580 markedly decreased the cell viability promoted by pcDNA3.1-Cdc42 (Fig. 4a). Additionally, the result of transwell assay showed that pcDNA3.1-Cdc42 promoted cell migration, while IWP-2 and SB203580 inhibited cell migration in SCs transfected with pcDNA3.1-Cdc42 ($P < 0.05$, Fig. 4b).

Discussion

Our finding indicated that expression of Cdc42 protein was markedly increased in SCs after sciatic nerve injury 7 days. Cdc42 promoted SCs proliferation and migration after

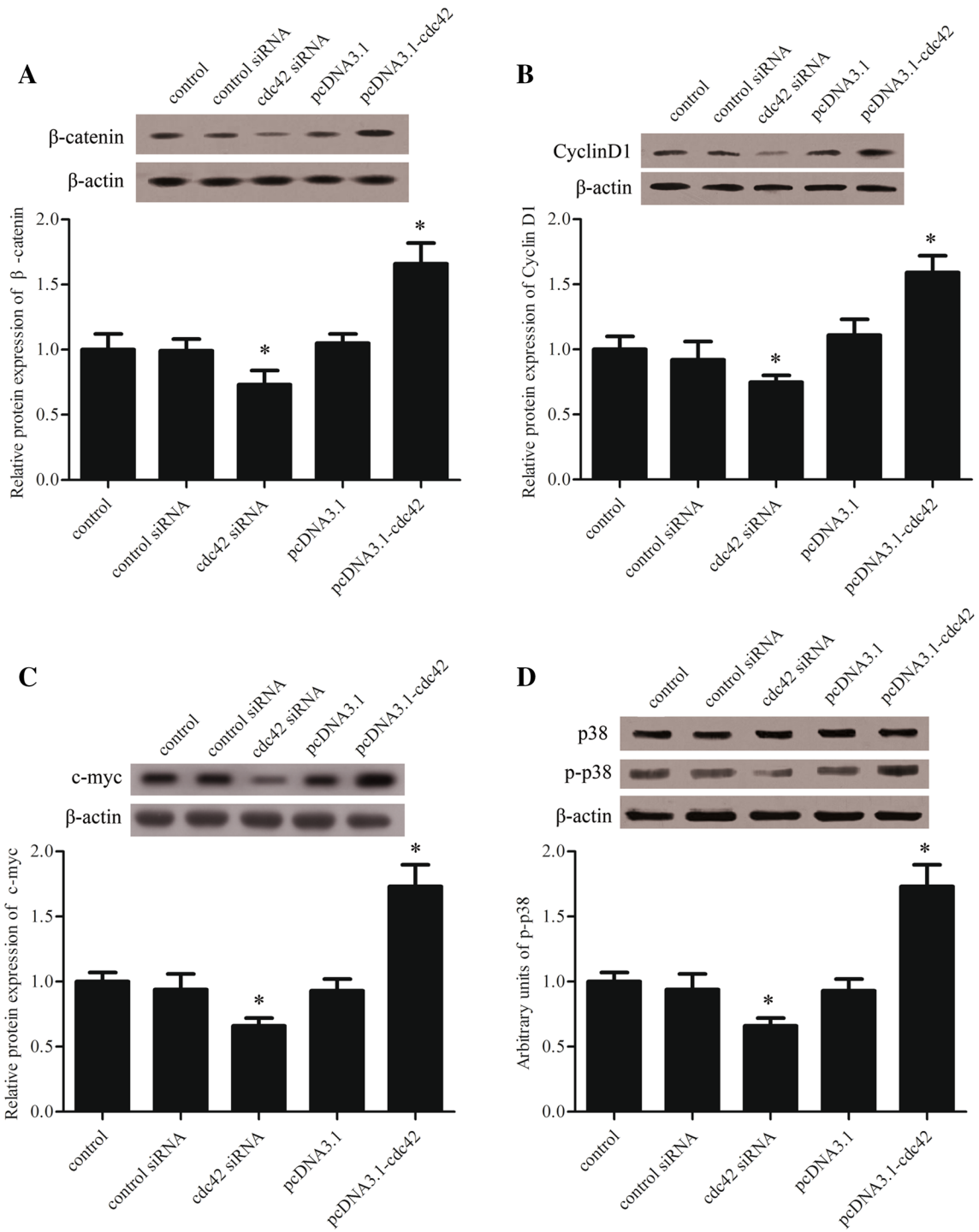

Fig. 3 The Effect of Cdc42 on Wnt/β-catenin and p38 MAPK signaling pathway in the sciatic nerves after injury. SCs isolated from sciatic nerves after injury for 7 days in SD rats were cultured and were transfected with control siRNA, Cdc42 siRNA, pcDNA3.1 and pcDNA3.1-Cdc42. The **a** β-catenin, **b** Cyclin D1, **c** c-myc, **d** the levels of p38 and p-p38 protein expression were measured by the western blot assay. Results are expressed as the mean ± SD. Each experiment was performed in triplicate. *$P < 0.05$ vs. control

sciatic nerve injury via activating Wnt/β-catenin and p38 MAPK signaling pathway.

Cdc42 protein contains two kinds of molecular structure, activation and non-activated state. These two molecular structures may occur a rapid conversion to contribute to cell migration as similar as a molecular switch and perform important regulatory function [9, 10]. This structures guide Cdc42 protein to act multiple complicated role in

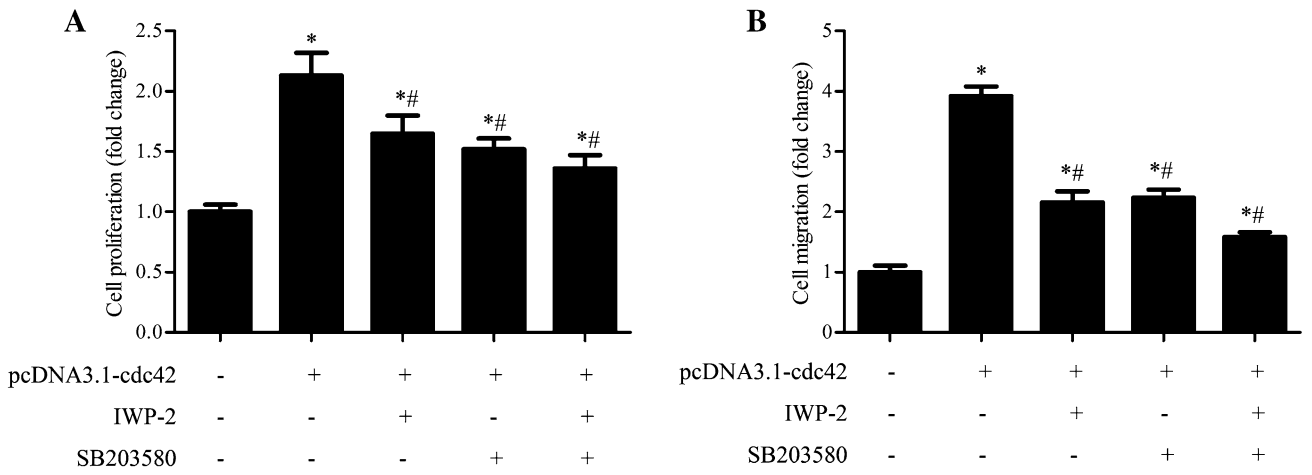

Fig. 4 The involvement of Wnt/β-catenin and p38 MAPK signaling in the Cdc42 mediated Schwann cell proliferation and migration in the injured sciatic. SCs isolated from sciatic nerves after injury for 7 days in SD rats were cultured and were treated with pcDNA3.1-Cdc42, Wnt/β-catenin signaling pathway inhibitor IWP-2 and p38 MAPK signaling pathway inhibitor SB203580. **a** Cell proliferation was investigated using Cell-LightTM EdU DNA Cell Proliferation Kit. **b** Cell migration was detected by transwell-based assay. Results are expressed as the mean ± SD. Each experiment was performed in triplicate. $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. pcDNA3.1-Cdc42

progression of cellular activity. Cdc42 acts downstream of the Wnt/Ca^{2+} signaling pathway to activate Par6/PKC complex [11, 12], regulating microtubule cytoskeleton and participating in the polarity formation and reorientation of centrosome [13]. The β-catenin is typically viewed as a central mediator of complex Wnt signaling pathway [14]. This study also indicated that Cdc42 knockdown inhibited β-catenin expression in SCs after sciatic nerve injury, indicating that Cdc42 could modulate β-catenin-dependent Wnt signaling. However, within the past 15 years, at least three Wnt-mediated pathways have been proposed that function independent of β-catenin, which include activation the Jun-N-terminal kinase (JNK) [14]. The study of Yamauchi J et al. had demonstrated that Cdc42 activated JNK cascade in SCs [15]. In fact, there is a close relationship between the p38 MAPK pathway and JNK pathway, JNK may be upstream of p38 and downstream of MAPK [16]. Therefore, activation of p38 MAPK pathway by Cdc42 may be connected with JNK pathway. Our finding suggested that Cdc42 knockdown suppressed activation of p38 MAPK pathway which seemed to be associated with β-catenin-independent Wnt signaling (JNK pathway). Overall, regulation of Cdc42 on SCs proliferation and migration after sciatic nerve injury perhaps not only through activation of β-catenin-dependent Wnt signaling but also β-catenin-independent Wnt signaling.

Cdc42 protein also modulate cell proliferation and migration by multiple other signaling pathway. Normally, only the Cdc42 protein located in front edge region of cell motility is activated, which is the regional activation mechanism of Cdc42 protein [17]. According to this mechanism, cytoskeletal components, such as microtubules, microfilament and so on, exhibit polarity distribution, which directly determines the direction of cell migration [18, 19]. Studies have shown that the external signal caused regional activation of Cdc42 protein mainly by regulating guanine nucleotide exchange factor (GEF) to thereby induce cell migration. In some external stimuli-induced cell migration, phosphorylated nudel protein by extracellular signal-regulated kinase (Erk) competitive combined GTPase activating protein(GAP) with Cdc42 protein in Cdc42-GAP complex, which leading to Cdc42 activation by GEF continued to maintain an activated state [20]. On the other hand, excessive activated Cdc42 protein competitively bound to nudel proteins to inhibit the activity of Cdc42-GAP and avoid excessive production of activated Cdc42 [20]. Furthermore, Cdc42 protein modulated activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, thus affecting cell migration [21, 22]. Study demonstrated that Cdc42 was located at the dorsal root ganglion (DRG) neurons, axons and SCs of the sciatic nerve, and mediating some intracellular signals of the peripheral nervous system by Rac/Cdc42-pak signaling pathway [5]. These finding indicated that regulation of Cdc42 on SCs proliferation and migration after sciatic nerve injury perhaps by multiple signaling pathway.

In conclusion, we show here that expression of Cdc42 was significantly increased in SCs after sciatic nerve. Overexpression of Cdc42 promoted SCs proliferation and migration after sciatic nerve injury via activating Wnt/β-catenin and p38 MAPK signaling pathway. Otherwise, Cdc42 knockdown inhibited SCs proliferation and migration via inactivating Wnt/β-catenin and p38 MAPK signaling pathway. Meanwhile, Wnt/β-catenin and p38 MAPK signaling

pathway were implicated in regulation of Cdc42 on SCs proliferation and migration after sciatic nerve injury.

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Compliance with Ethical Standards

Conflict of interest None.

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