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*Original Article*

# PDGF-BB activated Rac1 in regulation of inner and outer nuclear distribution of rat aortic smooth muscle cell β-catenin

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**Abstract:** Objective: In this study, we observed the interaction between JNK and Rac1 and its effect on PDGF-BB induced ß-catenin inner and outer nuclear distribution, cell proliferation and migration. Methods: SD rats' thoracic aortas were taken and rats' aortic smooth muscle cells were cultured by explant method. Cell morphology and immunohistochemistry were relied on to identify the cell. Three pairs of Rac1siRNA were designed. Western blotting method was used for screening, and the most effective one was applied to the experiment. CCK8 kits and Transwell chambers were used to detect the effects of different concentrations of Rac1 inhibitor NSC23766 (25, 50, 100 μmol/L) and Rac1siRNA (50 nmol/L) on vascular smooth muscle cells' proliferation and migration which was induced by PDGF-BB (50 μg/L). Results: The vascular smooth muscle cells grew out 5-7 days after explant method culture. The cell morphology and alpha -actin immunohistochemistry identified them as vascular smooth muscle cells. After 24 h PDGF-BB stimulation, vascular smooth muscle cell cultured CCK8 was significantly increased (OD, group NSC23766: 0.796+0.083 VS 0.371+0.055, P<0.01; group Rac1siRNA: 0.948+0.107 VS 0.443+0.042, P<0.01). But if given different concentrations of NSC23766 (25, 50, 100 mol/L) before treatment, the concentration of CCK8 decreased dependently (OD: 0.796+0.083 VS 0.559+0.064, P<0.05; 0.490+0.027, 0.370+0.025, P<0.01). CCK8 value was also decreased significantly (0.948+0.107 VS 0.670+0.072, P<0.01) after transfection of Rac1siRNA (50 nmol/L). Migrations of vascular smooth muscle cells were significantly increased after PDGF-BB stimulation (crystal violet OD, group NSC23766: 0.429+0.0400 VS 0.260+0.023, P<0.01; group Rac1siRNA: 0.439+0.042 VS 0.300+0.042, P<0.01). Cell migration was inhibited obviously (OD: 0.429+0.040 VS 0.401+0.015, P>0.05, 0.324+0.035, 0.290+0.013 and 0.439+0.042, P<0.01 VS 0.345+0.030, P<0.05) if they were cultured with different concentrations of NSC23766 (25, 50, 100 mol/L) and Rac1siRNA (50 nmol/L). Rac1 activity, expression of pi-JNK and cell nucleus beta -catenin were gradually increased after stimulation of PDGF-BB and reached its peak in 5 min, 15 min and 60 min. With different concentrations of NSC23766 (25, 50, 100 mol/L), SP600125 (10, 20, 40 g/L) and Rac1siRNA (50 nmol/L) treatment and the same time of PDGF-BB stimulation, the expression activity of Rac1, pi-JNK and nucleus beta -catenin were inhibited obviously and showed concentration dependent. Immunofluorescence results showed: After 60min PDGF-BB stimulation on vascular smooth muscle cell, Beta -catenin nuclear accumulation was obvious. When they were given NSC23766 (100 mol/L), SP600125 (40 g/L) and Rac1siRNA (50 nmol/L) treatment, this aggregation was inhibited. Conclusion: The interaction existed between the Rac1 activation and JNK phosphorylation, and it played a key regulatory role in PDGF-BB induced vascular smooth cell beta -catenin nuclear cohesive concentration. Thereby it regulated vascular smooth muscle cells' proliferation and migration which were induced by PDGF-BB.

**Keywords:** PDGF-BB, Rac1, aortic smooth muscle cell, β-catenin

## Introduction

Rac1 belongs to the Rho GTP enzyme family. Previous reports showed that Rac1 activation played an important role in tumorigenesis, invasion, metastasis, cell cycle regulation and apoptosis [1]. In addition, in some cell lines, PDGF-BB played an important role in its movement and proliferation by activating G-protein Rac1 and Cdc42 [2]. ß-catenin is a cytoskeletal protein. It was encoded by *ctnnbl* gene which located on chromosome 3p21-22. After the

activation of cytokines, ß-catenin entered into the nucleus and activated the transcription factor causing cell proliferation. It has been reported that it played an important role in the proliferation of vascular smooth muscle cells [3]. ß-catenin entered into the nucleus by the classic Wnt activation, which was also regulated by JNK, AKT, E-cadherin and other molecules.

It has been reported previously that PDGF-BB activated Rac1 and promoted aggregation of ß-catenin in the nucleus and played an important role in vascular smooth muscle cells proliferation and migration [4-6]. Wu et al. [7] reported that the activation of Rac1 and JNK promoted ß-catenin entered into the nucleus, regulated cell proliferation and embryonic limbs growth. However, whether Rac1 activation played a regulatory role in ß-catenin nucleus gathering induced by PDGF-BB remained to be unknown. Therefore, this study investigated mutual adjustment between Rac1 and the activation of JNK, its effect on the inner and outer distribution of ß-catenin induced by PDGF-BB and its role in vascular smooth muscle cells' proliferation and migration.

## Material and methods

### *Experimental animals*

SD male rats were purchased from the animal center of Luzhou Medical College, weighting 120-160 g.

### *Isolation, culture and identification of rat aortic smooth muscle cells*

Take one SD rat and intraperitoneal inject sodium pentobarbital (65 mg/kg) to anesthetize the rat. Open the chest and isolate the aorta. Except film and endometrium immediately in sterile conditions. Smooth membrane grassroots were cut into 1-2 mm blocks, and then posted on 25 $cm^2$ flasks. Invert the flask, and add DMEM medium 5 ml L containing 20% fetal bovine serum and 100u/penicillin and streptomycin. They were incubated at 37°C, 5% $CO_2$ incubator. Gently flip the flask in order to make the culture cover tissue block after 2 hours incubation. Then continue to culture at 37°C, 5% carbon dioxide incubator for 3-5 days to observe cells grown situation. When the cells were covered, take trypsin digestion and passage. 3-8 passage cells were used in experiment. The cultured primary cells were placed under inverted phase contrast microscope and observed at different time points and took photograph.

### *siRNA interference*

Rat Rac1 Gene sequences were found in GeneBank (GenBank number was NM_134366.1) and entered into the RNAi design software; three rat Rac1 sequences with the highest specific score were selected, and the sequences were as follows:

siRNA1: Sense strand: 5'-GCUUGAUCUUAGGGAUGAUdTdT-3'; Antisense strand: 3'-dTdTCGAACUAGAAUCCCUACUA-5'.

siRNA2: Sense strand: 5'-GACUCAAGACAGUGUUUGA dTdT-3'; Antisense strand: 3'-dTdT CUGAGUUCUGUCACAAACU-5'.

siRNA3: Sense strand: 5'-CAAACAGACGUGUUCUUAA dTdT-3'; Antisense strand: 3'-dTdT GUUUGUCUGCACAAGAAUU-5'.

The above siRNA and the unknown control siRNA were synthesized by Guangzhou Rui Bo Biological Technology Co., Ltd., respectively 5 nmols. Groups were as follows: normal control group, unknown control siRNA (NsiRNA) group, Rac1siRNA1 group, Rac1siRNA12 group, and Rac1siRNA3 group.

siRNA interference was as follows: Cells of 5th to 10th generations were seeded in six-well plates; when reaching 50-70% confluence, cells were washed twice with PBS at 24 hours before interference and incubated with 1.5 ml antibiotic and serum-free DMEM medium. 5 μl aforementioned annealing buffer containing siRNA was mixed with 250 μl Opti-MEM-I; 5 μl LipofectamineTM2000 and 250 μl Opti-MEM-I were added into another centrifuge tube, incubating at room temperature for 5 to 10 minutes. The two solutions were mixed using reverse method (the volume was 500 ul); after incubated at room temperature for 20 to 30 minutes, the solution became cloudy. 500 μl siRNA-LipofectamineTM2000 was added into the antibiotic-free culture medium (1.5 ml), and the final siRNA concentration was 50 nmol/L. After 6 hours, antibiotic-free culture medium containing 10% fetal bovine serum was added to make the volume of each well reach 3 ml; the experiment was conducted two days later.

*Cell grouping*

(1) NSC23766 groups: serum-free medium group, PDGF-BB (50 μg/L) stimulation group, PDGF-BB (50 μg/L)+NSC23766 (25 μmol/L) group, PDGF-BB (50 μg/L)+NSC23766 (50 μmol/L) group, PDGF-BB (50 μg/L)+NSC23766 (100 μmol/L) group.

(2) Rac1siRNA groups: serum-free medium group, PDGF-BB (50 μg/L) stimulation group, PDGF-BB (50 μg/L)+Rac1siRNA (50 nmol/L) group.

(3) SP600125 groups: serum-free medium group, PDGF-BB (50 μg/L) stimulation group, PDGF-BB (50 μg/L)+SP600125 (10 μg/L) group, PDGF-BB (50 μg/L)+SP600125 (20 μg/L) group, PDGF-BB (50 μg/L)+SP600125 (40 μg/L) group.

*Detection of cell proliferation (CCK8 assay)*

The smooth muscle cells were seeded in 96 well plates at a density of $1\times10^4$ cells/well; after the adhesion, cells were incubated with serum-free medium for 24 hours. According to the above groups, cells were treated with NSC23766 (final concentration were 25, 50, 100 μmol/L) for 24 hours; after Rac1siRNA transfection for 6 h, cells were incubated with serum medium; after 16 h, medium was replaced with serum-free medium again. In addition to the control group, PDGF-BB (50 μg/L) was added in all groups; after 20 hours, 20 ul WST-8 (2- (2- methoxy-4-nitrophenyl) -3-(4-nitrophenyl)-5-(2,4-benzene disulfonate)-2H-tetrazolium, monosodium salt) was added; after cultured for 4 hours, OD values of each well were measured on the enzyme-linked immunosorbent instrument (450 nm). Each group set up three parallel wells.

*Transwell assay to detect smooth muscle cell migration*

After trypsinization, 10% FBS was added to terminate digestion; cells were collected in a 15ml centrifuge tube and centrifuged; serum-free medium was added to adjust the cell concentration to $5 \times 10^5$/ml. 500 ul serum-free medium was added into the lower room of transwell chamber; according to the aforementioned groups (each group with 3 wells), PDGF-BB (50 μg/L) was added in the corresponding lower room; insert plate was inserted and 200ul cell suspension was added into each plate (in Rac1siRNA group, cells were directly digested after culture for 24 hours and added into the dish); as the previous grouping, corresponding concentrations of NSC23766 were added to each plate, incubating (37°C, 5% $CO_2$ and saturated humidity) for 24 h. The insert dish and the medium were removed; cells in the upper plate were gently wiped with a cotton swab; the lower plate was washed twice with PBS, and residual liquid was exhausted; methanol was added to fix cells for 20 min. Fixative liquid was discarded; cells were stained with 0.1% crystal violet for 15 min, and then washed with PBS. Stained cells were observed under an inverted microscope and photographed. The stained cells in insert dish were washed with 500 ul 50% ethanol containing 0.1 mol/L citrate sodium; 200 ul wash liquid was transferred into 96-well plates; crystal violet absorbance (A) of each well at 585 nm was measured on the enzyme-linked immunosorbent instrument, and the average was calculated.

*Cell processing and protein extraction*

(1) The time points for each protein after PDGF-BB stimulation:

a: Rac1: Respectively at 0 min, 1 min, 5 min, 15 min, 30 min, 60 min after PDGF-BB stimulation, total protein was extracted.

b: JNK: Respectively at 0 min, 5 min, 15 min, 30 min, 60 min and 120 min after PDGF-BB stimulation, total protein was extracted.

c: β-catenin: Respectively at 0 min, 15 min, 30 min, 60 min, 120 min after PDGF-BB stimulation, total protein was extracted.

(2) Cells were incubated with serum-free medium for 24 h, and incubated with gradient concentrations of SP600125 (10, 20, 40 μg/L) and NSC23766 (25, 50, 100 μmol/L) for another 24 h; and them vascular smooth muscle cells were transfected with Rac1siRNA for 40 h before serum-free culture for 8 h. Then PDGF-BB stimulation was given and proteins were extracted at the following time points:

Rac1: 5 min, total protein; JNK, pi-JNK: 15 min, total protein; β-catenin: 1 h, cytoplasmic - nuclear protein.

*Western blotting*

Western blotting was performed according to the method of literature. Each antibody was

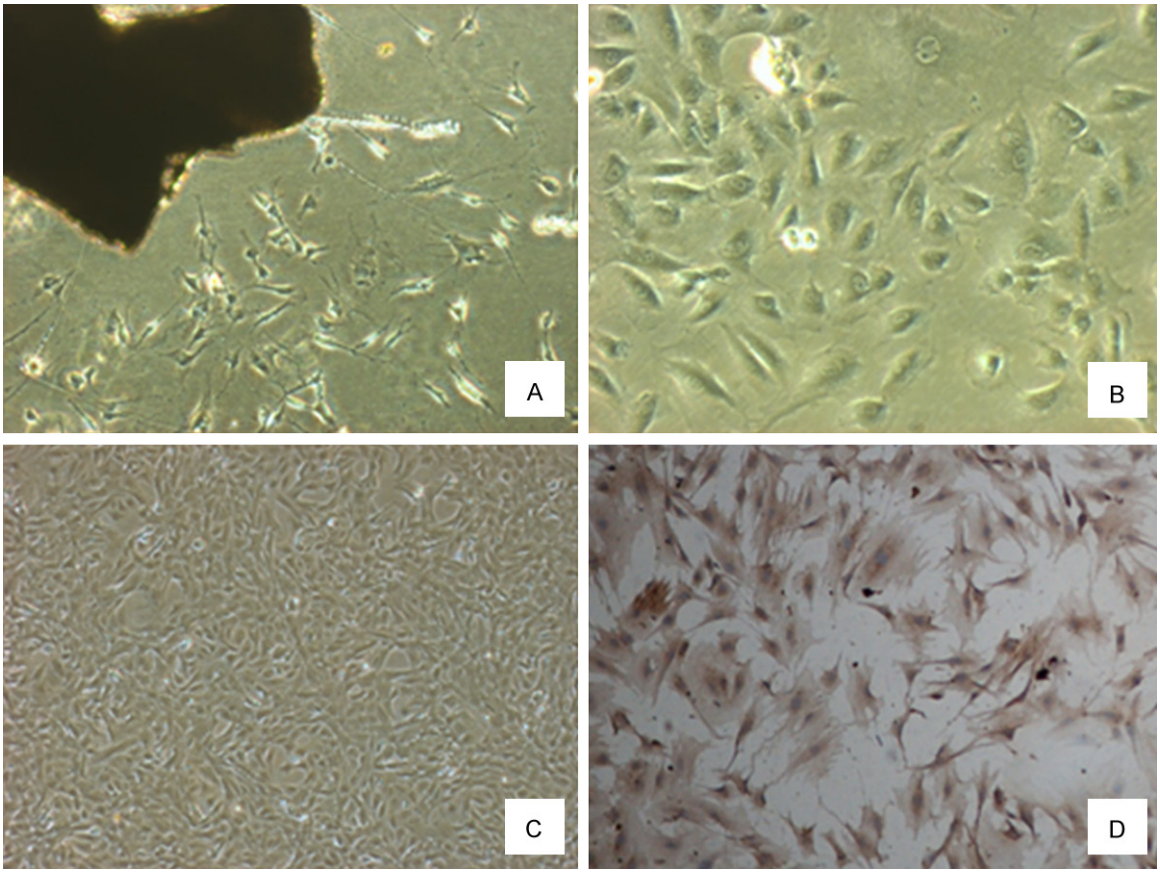


**Figure 1.** Culture and identification of rat aortic smooth muscle cells. A: In the first 5-7 days, vascular smooth muscle cells grew from the tissue blocks gradually, cells were small, polygonal and dough (×100). B: Cells gradually grew into spindle shape; when dense integrating, clustered growth was observed (×200). C: When cells fully fused, they exhibited typical characteristics of vascular smooth muscle cell growth, named "peaks and valleys" growth (×100). D: Immunohistochemistry results showed positive intracytoplasmic α-SMC-actin associated antigen expression (×200).

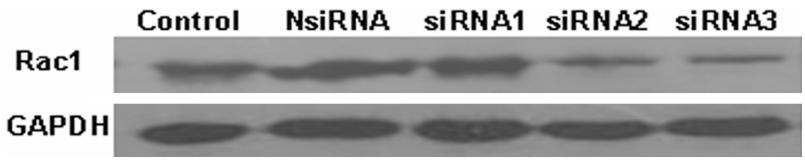


**Figure 2.** Rac1siRNA Screening: Western blot indicated the third pair of siRNA was the most effective.

diluted with TBST solution containing 5% non-fat dry milk: Rac1, JNK, pi-JNK and β-catenin (1:1000); cells were incubated with mouse anti-rat GAPDH (1:4000) at 4°C overnight. Primary antibody rewarmed at room temperature for 1 hour before TBS washing membrane (10 min×3 times), then the corresponding horseradish peroxidase-conjugated secondary antibody (1:5000) was added, incubated at room temperature for 1 h; the membrane was washed with TBST (10 min×2 times) and TBS (1 times). In darkroom, ECL luminescent liquid was dropped on the membrane; tableting was performed with film covered, and the film was placed in the developer; until the developing stripe was clear, it was placed in fixer for fixing; and then protein bands were scanned. Quatity one software was used to analyze the gray value of each protein band. GAPDH was used as internal control, and the gray value ratio of the target protein and GAPDH bands represented the relative content of the target protein; result analysis was performed.

*GST-pulldown to detect Rac1 activity*

The operation was carried out according to the Rac1 activity assay kit.

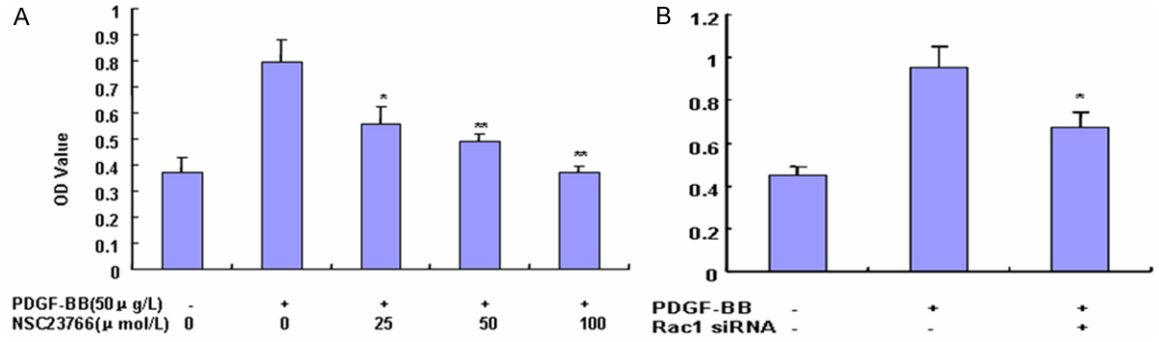


**Figure 3.** Effect of NSC23766 and Rac1siRNA on PDGF-BB induced proliferation. A: NSC23766; B: Rac1siRNA. (*P<0.05;**P<0.01, vs. PDGF-BB group, n=3).

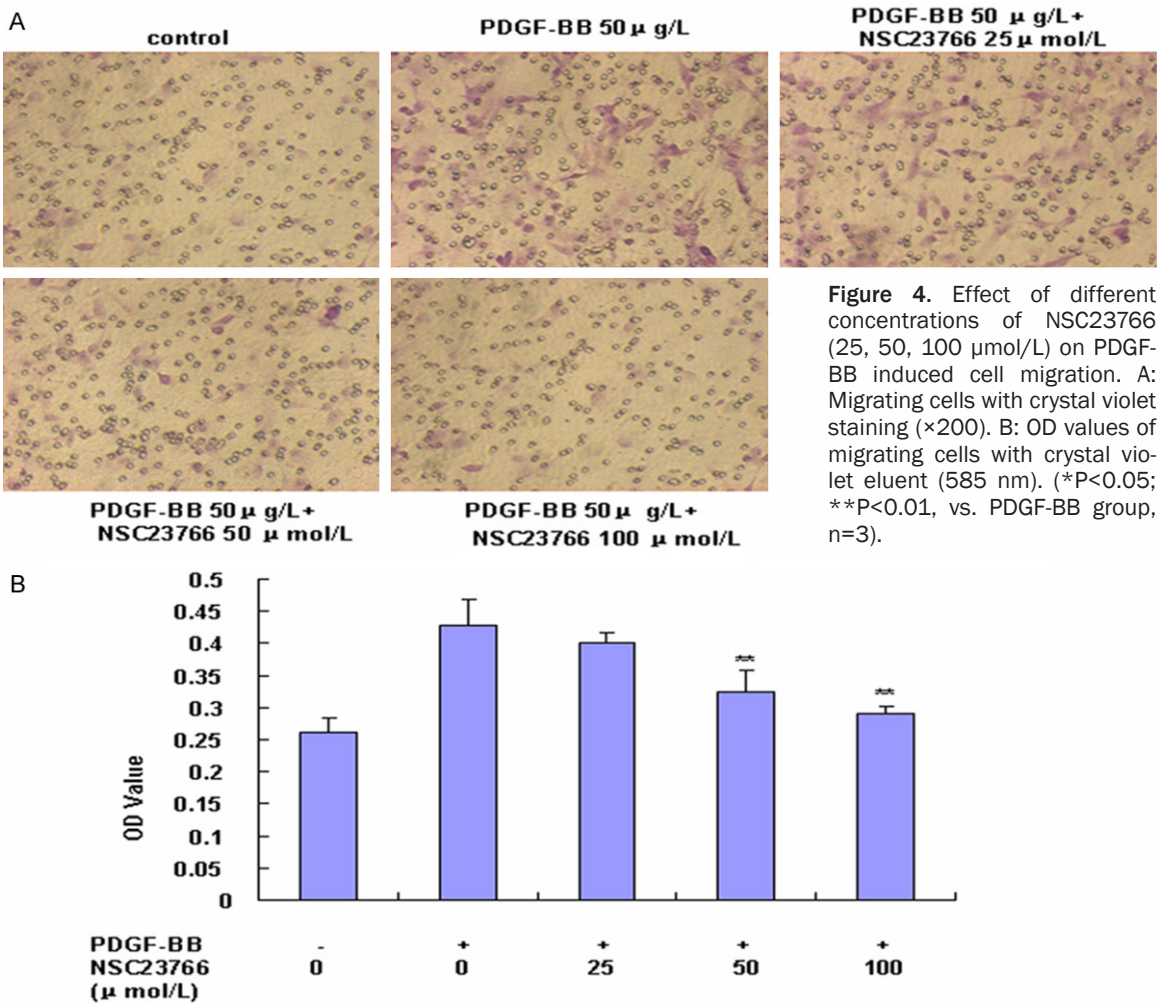


**Figure 4.** Effect of different concentrations of NSC23766 (25, 50, 100 μmol/L) on PDGF-BB induced cell migration. A: Migrating cells with crystal violet staining (×200). B: OD values of migrating cells with crystal violet eluent (585 nm). (*P<0.05; **P<0.01, vs. PDGF-BB group, n=3).

*Immunofluorescence detection of β-catenin nuclear distribution*

The coverslip (13 mm) was soaked in acid, washed and sterilized by high pressure; then 1% gelatin was added into 24 well plate for overnight incubation; after PBS rinsing, the medium was added and the vascular smooth muscle cells were seeded in 24 well plate with coverslip. Vascular smooth muscle cells were incubated with serum free culture for 24 h, and then incubated with SP600125 (40 g/L) and

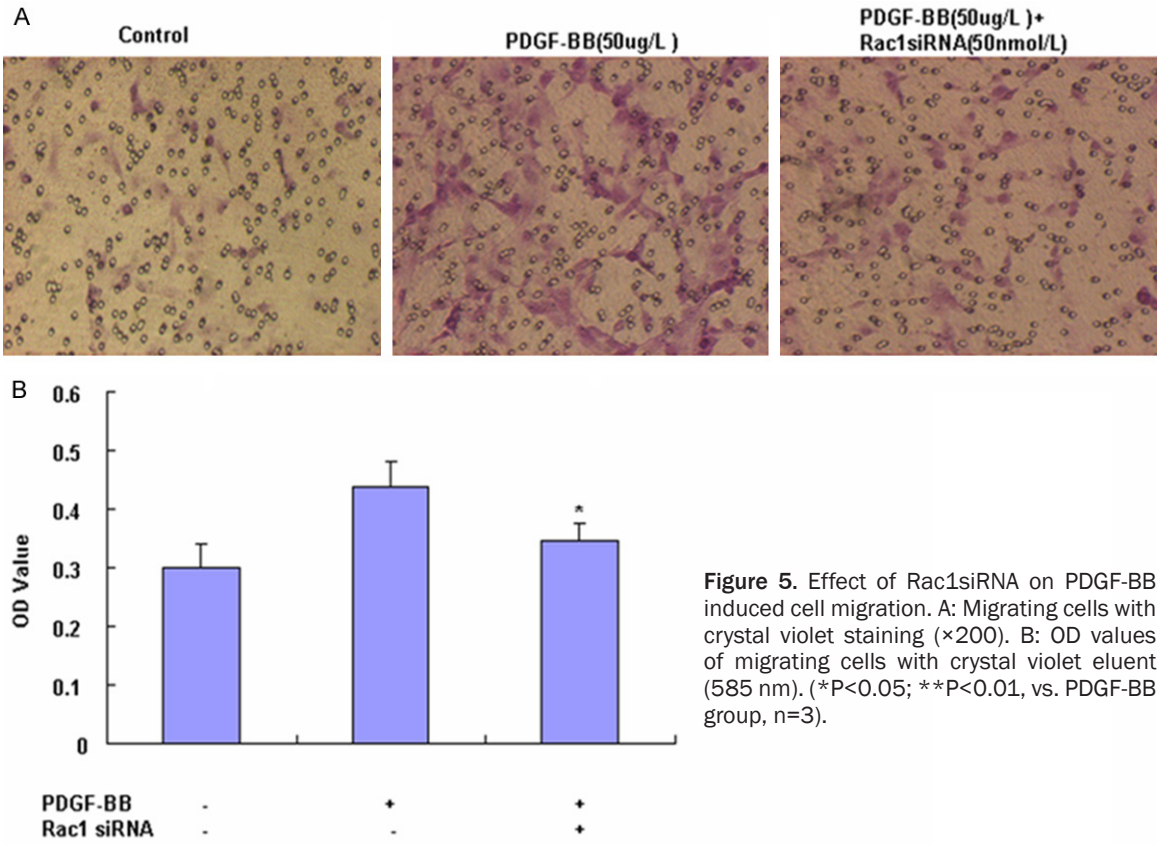


**Figure 5.** Effect of Rac1siRNA on PDGF-BB induced cell migration. A: Migrating cells with crystal violet staining (×200). B: OD values of migrating cells with crystal violet eluent (585 nm). (*P<0.05; **P<0.01, vs. PDGF-BB group, n=3).

NSC23766 (100 mol/L) for 24 h; After 40 h of Rac1siRNA (50 nmol/L) transfection, cells were incubated with serum-free culture medium for 8 h, and then stimulated by PDGF-BB for 1 h.

The cell climbing piece was removed from 24 well plate, washed with PBS (5 min×3), and fixed in 4% paraformaldehyde for 20 min; then it was transparent by 0.2% Triton X-100 for 20 min at room temperature, blocked with 5% goat serum for 30min, incubated with Rabbit anti rat β-catenin antibody (1:100) at 4°C overnight, and incubated with Goat anti rabbit IgG (H&L)-Rhodamine secondary antibody (1:100) at room temperature for 2 hours in dark. DAPI staining and anti-fluorescence decay mounting were performed. At last, the sample was observed under the fluorescence microscope and photographed. PBS washing was performed 3 times after every operation.

## Results

*Culture and identification of vascular smooth muscle cells*

Inverted fluorescence microscope was used to observe cell morphology, as shown in **Figure 1**.

*Screening for Rac1siRNA interference*

After 48 hours of interference, total protein was extracted in the control group, NsiRNA group, Rac1siRNA1, siRNA2 and siRNA3 groups; the expression of Rac1 was detected by western blot; the results showed that: no significant difference had been found in the expression of Rac1 between the control group and NsiRNA group; Rac1 expression slightly decreased in siRNA1 group compared with the control group; the expression of Rac1 significantly decreased in siRNA2 and siRNA3 groups, and the decline in siRNA3 group was more significant. Therefore, this experiment selected the third pair of Rac1siRNA (sense strand: 5'-CAAAC-AGACGUGUUCUUAA dTdT-3'; antisense strand: 3'-dTdTGUUUGUCUGCACAAGAAUU-5') as experimental siRNA (**Figure 2**).

*Effect of NSC23766 on PDGF-BB induced proliferation*

After stimulated by 50 μg/L PDGF-BB for 24 h, the CCK8 absorbance values of smooth muscle cells increased to 214% of those in the control group (0.796±0.083 VS 0.371±0.055, P<0.01).

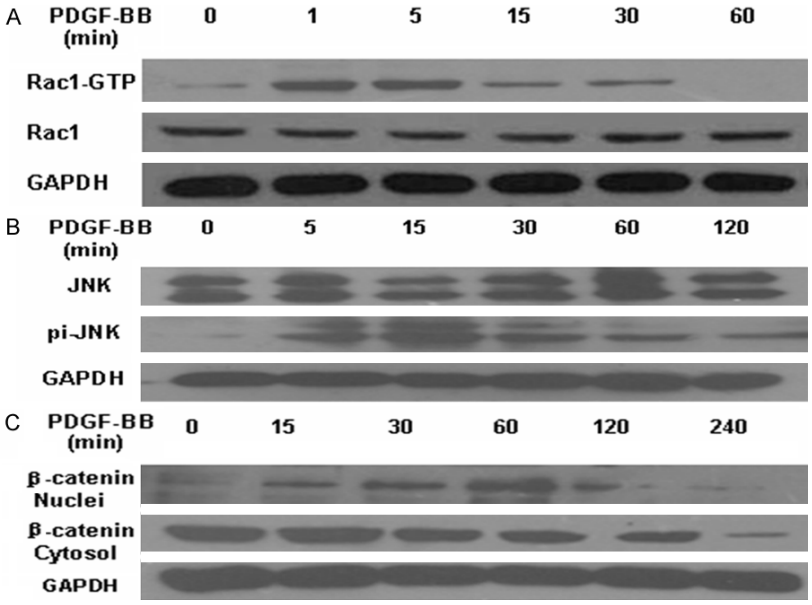


**Figure 6.** A: Time characteristics of Rac1 activity after PDGF-BB stimulation; Rac1 activation peaked at 5 min. B: Time characteristics of pi-JNK expression after PDGF-BB stimulation; pi-JNK expression reached a peak at 15 min. C: Time characteristics of cytoplasmic and nuclear β-catenin expression after PDGF-BB stimulation. β-catenin accumulation in the nucleus peaked at 60 min.

After treated with gradient concentrations (25, 50, 100 μmol/L) of Rac1 inhibitor NSC23766 for 24 hours and 50 μg/L PDGF-BB for another 24 hours, CCK8 absorbance values showed concentration gradient-dependent decrease (0.559±0.064, 0.490±0.027, 0.370±0.025 VS 0.796±0.083, P<0.01) (**Figure 3A**).

*Effect of Rac1siRNA on PDGF-BB induced proliferation*

After stimulated by PDGF-BB (50 μg/L), CCK8 absorbance values were significantly increased (0.948±0.107 VS 0.443±0.042, P<0.01). But after Rac1siRNA (50 nmol/L) transfection and PDGF-BB stimulation, CCK8 absorbance values were significantly lower than that in PDGF-BB stimulation group (0.948±0.107 VS 0.670± 0.072, P<0.05) (**Figure 3B**).

*Effect of NSC23766 on PDGF-BB induced migration*

After PDGF-BB (50 μg/L) stimulating vascular smooth muscle cells for 24 hours, compared to the control group, Transwell chamber assay showed that cell migration was significantly increased (crystal violet OD value: 0.429±0.040 VS 0.260±0.023, P<0.01); the upper chamber was treated with gradient concentrations (25, 50, 100 μmol/L) of Rac1 inhibitor NSC23766, and the lower chamber was treated with 50 μg/L of PDGF-BB for 24 hours; and then Transwell chamber showed a concentration gradient-dependent decrease in migrated vascular smooth muscle cells (compared to PDGF-BB stimulation group) (OD value: 0.401±0.015, 0.324± 0.035, 0.290±0.0130 VS 429±0.0400) (**Figure 4**).

*Effect of Rac1siRNA on PDGF-BB induced migration*

After vascular smooth muscle cells received PDGF-BB (50 μg/L) stimulation for 24 hours, cell migration experiments showed that the number of migrating cells increased significantly; the absorbance of eluted crystal violet at 585 nm was significantly higher (0.439±0.042 VS 0.300± 0.042, P<0.01). In Rac1siRNA transfected VSMCs, migration was inhibited significantly (0.439±0.042 VS 0.345±0.030, P<0.05) (**Figure 5**).

*Time characteristics of PDGF-BB-induced Rac1 activation, pi-JNK expression and β-catenin accumulation in the nucleus*

Vascular smooth muscle cells were cultured in serum-free medium for 24 hours, and then stimulated with 50 μg/L PDGF-BB; Rac1 activity was detected respectively at 1 min, 5 min, 15 min, 30 min and 60 min after stimulation by GST-pulldown assay.

The results suggest that: after PDGF-BB stimulation, Rac1 activity gradually increased, reached a peak in 5 min and then gradually decreased (**Figure 6A**). In 5 min, 15 min, 30 min, 60 min and 120 min after 50 μg/L PDGF-BB stimulation on VSMC, western blot was used to detect the expression of JNK and pi-JNK, and it showed that after PDGF-BB stimulation, pi-JNK expression gradually increased, peaked in 15 min, and then gradually decreased (**Figure 6B**). At 5 min, 15 min, 30 min, 60 min,

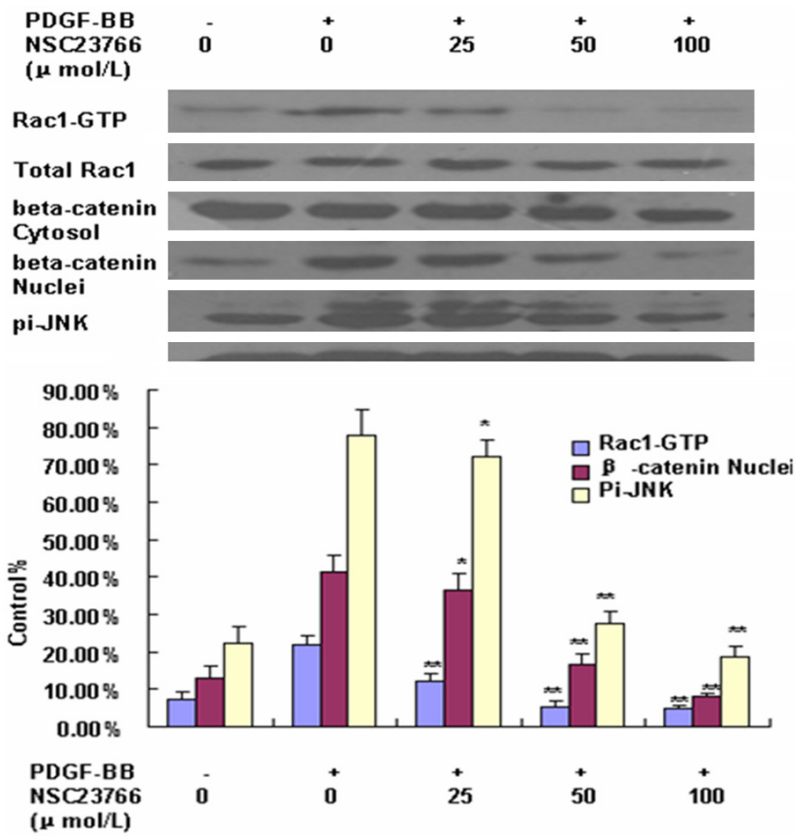


**Figure 7.** Effect of different concentrations of NSC23766 (25, 50, 100 μmol/L) on PDGF-BB-induced Rac1 activation, pi-JNK expression, and β-catenin nuclear accumulation (*P<0.05; **P<0.01 , vs. PDGF-BB group, n=3).

120 min and 240 min after 50 μg/L PDGF-BB stimulation, cytoplasm-nucleus protein was isolated, and western blotting detection of β-catenin was performed; the results showed: β-catenin in the nucleus gradually increased after stimulation, reached a peak at 60min, and then gradually decreased. The cytoplasmic β-catenin was gradually decreased (**Figure 6A-C**).

*Effect of NSC23766 on PDGF-BB-induced Rac1 activation, pi-JNK expression and β-catenin nuclear accumulation*

Rac1 activity was significantly increased at 5 min after PDGF-BB stimulation (7.42%±2.02% VS 21.71±2.51%, P<0.01). After pretreatment of different concentrations of Rac1 inhibitors NSC23766 (25, 50, 100 μmol/L), compared with PDGF-BB stimulation group, Rac1-GTP decreased in concentration gradient-dependent manner, respectively reduced 44.27%, 75.96% and 78.35%. pi-JNK expression and β-catenin expression in the nucleus were significantly increased at 15 min (22.47±4.10% VS 78.03±6.59%, P<0.01) and 60 min (12.79±3.52% VS 41.52±4.42%, P<0.01) after PDGF-BB stimulation. After pretreatment with different concentrations of Rac1 inhibitor NSC23766 (25, 50, 100 μmol/L), compared to PDGF-BB stimulation group, both the two showed a concentration gradient dependent decrease, and respectively decreased: 7.34%, 64.58%, 76.21% (pi-JNK) and 12.24%, 59.49%, 80.60% (the nucleus β-catenin), (**Figure 7**).

*Effect of SP600125 on PDGF-BB-induced Rac1 activation, pi-JNK expression and β-catenin nuclear accumulation*

Rac1 activity significantly increased at 5 min after PDGF-BB stimulation (5.46 ±2.18% VS 57.05±6.50%). After pretreatment with different concentrations of JNK inhibitor SP600125 (10, 20, 40 μg/L), compared with PDGF-BB stimulation group, Rac1-GTP showed a concentration gradient dependent decrease, and decreased 23.75%, 60.11% and 84.93% respectively. pi-JNK expression and β-catenin expression in the nucleus were significantly increased at 15 min (32.28±6.44% vs. 99.82%±6.50) and 60 min (5.23±1.25% vs. 59.93±6.55%) after PDGF-BB stimulation. After pretreatment with different concentrations of JNK inhibitor SP600125 (10, 20, 40 μg/L), compared to PDGF-BB stimulation group, both the two showed a concentration gradient dependent decrease, and respectively decreased: 22.14%, 44.77%, 78.79% (pi-JNK) and 36.11%, 44.95%, 89.37% (the nucleus β-catenin), (**Figure 8**).

*Effect of Rac1siRNA on PDGF-BB-induced Rac1 activation, pi-JNK expression and β-catenin nuclear accumulation*

No significant difference was found in total Rac1 between PDGF-BB stimulation group and

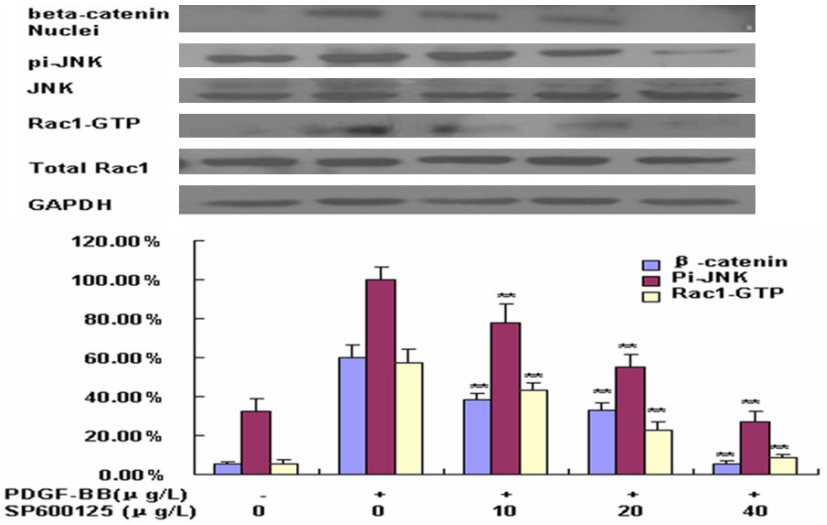


**Figure 8.** Effect of different concentrations of SP600125 (10 μg/L, 20 μg/L, 40 μg/L) on PDGF-BB-induced Rac1 activation, pi-JNK expression and β-catenin nuclear accumulation (*P<0.05; **P<0.01, vs. PDGF-BB group, n=3).

the control group, but in Rac1siRNA (50 nmol/L) group, Rac1 expression was significantly decreased. The GST pulldown detection of active Rac1 expression showed: at 5 min after PDGF-BB stimulation, Rac1-GTP expression was significantly increased, while in Rac1siRNA group, its expression had been significantly inhibited. At the same time, the expression of pi-JNK and β-catenin expression in the nucleus were also examined, and the results showed: at 15 min and 60 min after PDGF-BB stimulation, pi-JNK expression and β-catenin expression in the nucleus were significantly increased, but in Rac1siRNA interference group, their expression was significantly inhibited (**Figure 9**).

*Immunofluorescence for the effect of NSC23766, SP600125 and Rac1siRNA on PDGF-BB-induced β-catenin nuclear accumulation*

In order to further confirm the effect of Rac1 inhibitor NSC23766 (100 μmol/L), JNK inhibitor SP600125 (40 μg/L) and Rac1siRNA (50 nmol/L) on PDGF-BB-induced β-catenin nuclear aggregation, immunofluorescence detection was performed (DAPI stained nuclei). The results showed that: After giving PDGF-BB stimulation for 1 h, β-catenin gathered significantly in the vascular smooth muscle cell nuclei. However, after pre-treatment with NSC23766 (100 μmol/L), SP600125 (40 μg/L) and Rac1siRNA (50 nmol/L), and after PDGF-BB stimulation, β-catenin aggregation in the nucleus was inhibited significantly, shown in **Figure 10**.

## Discussion

The full name of Rac1 is Ras related C3 botulinum toxin substrate 1; it is located in the cell membrane, which belongs to the Rho-GTP enzyme family [8]. The Rho-GTP enzyme is a member of the small GTPases family, which is a group of monomeric GTP binding protein with molecular mass of about 20~25 kDa; its activity is regulated by the GDP/GTP circulation [9]. Rho protein binds to GDP in inactive state, then under the action of guanine nucleotide exchange factor (GEF) or GDP dissociation stimulator (GDS), GDP is released; hence it is activated when combining with GTP [10]. Activated Rho protein affects various signaling molecules in the cytosol to regulate intracellular signal transmission [11]. It has been previously reported that in smooth muscle cells and other cell lines, PDGF-BB played an important regulatory role in the proliferation and movement of G- protein Rac1 and Cdc42 by activating them [12, 13]. By CCK8 and Transwell chamber assay, the present study also showed that: PDGF-BB can promote the proliferation and migration of rat vascular smooth muscle cells, but after the administration of different concentrations of Rac1 inhibitor NSC23766, their proliferation and migration had obviously been suppressed and showed a concentration gradient-dependent decrease. We also examined the impact of Rac1 inhibitor NSC23766 on Rac1 activity, and found that it also showed a concentration gradient dependence. In order to detect the role of Rac1 activity in PDGF-BB-induced vascular smooth muscle cell proliferation and migration, Rac1siRNA interference was applied to the experiment, and the results showed that after Rac1siRNA transfection into vascular smooth muscle cells, Rac1 expression and activity were significantly reduced, meanwhile PDGF-BB-induced VSMC proliferation and

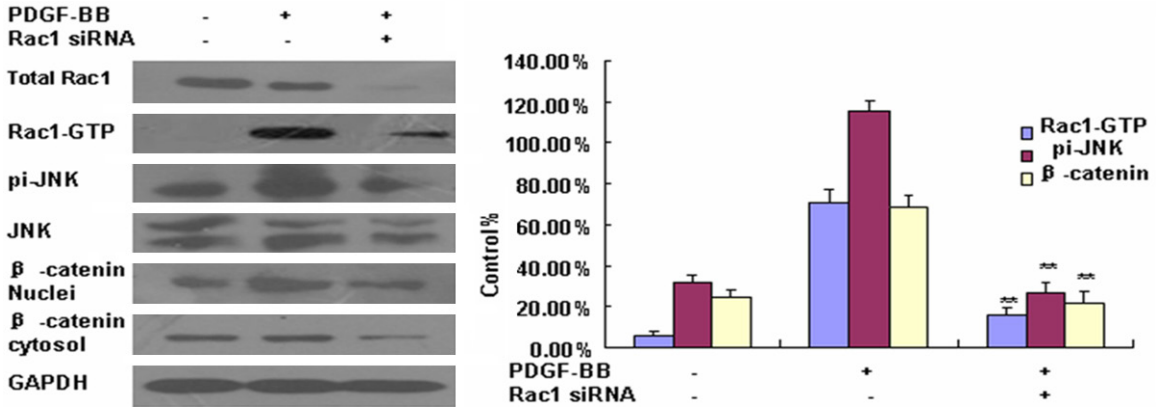


**Figure 9.** Effect of Rac1siRNA interference on PDGF-BB-induced Rac1 activation, pi-JNK expression, and β-catenin nuclear aggregation (*P<0.05; **P<0.01, vs. PDGF-BB group, n=3).

migration were inhibited significantly, which indicated that Rac1 played a crucial regulatory role in the proliferation and migration of vascular smooth muscle cells.

PDGF (platelet derived growth factor) is a known significant stimulating factor for abnormal vascular smooth muscle cells proliferation and migration. A few evidences suggested that PDGF-BB and PDGF receptor β mediated signaling pathway played a vital role in vascular remodeling and neointimal formation after vascular injury [2-4]. PDGFR activation is related with a series of tyrosine homologous region 2-related proteins,including: RasGAP, phosphatidylinositol (-3) kinase (PI3K) and phospholipase C (PLC) γ1; these molecules further induce specific signaling cascades to stimulate PDGFR downstream signals, such as ERK, AKT, JNK and small molecule protein rho and Rac1; these signals are involved in the PDGF-induced cell response: proliferation and migration [11, 12, 13]. Meanwhile it has been reported in the vascular smooth muscle cells and a variety of other cell lines that: PDGF-BB stimulation can promote ß-catenin transfer into the nucleus and gather in the nucleus, thus activating nuclear transcription factors and regulating the actin cytoskeleton, finally inducing cell proliferation and migration [1-3]. Above data suggest that Rac1 activation may play a very important role in PDGF-BB-induced ß-catenin accumulation in the nucleus of vascular smooth muscle cells.

To prove the possibility of such a regulatory role, the PDGF-BB-induced Rac1 activation, JNK phosphorylation and temporal characteristics of ß-catenin nuclear accumulation were firstly detected in this experiment. The results showed that: after PDGF-BB stimulation of VSMCs, Rac1 and pi-JNK activity and ß-catenin expression in the nucleus were gradually increased; Rac1 activity peaked in 5 min; pi-JNK and ß-catenin nuclear aggregation respectively reached a peak in 15 min and 60 min, and then gradually decreased. Temporal characteristics showed that: there may be a regulatory relationship among Rac1 and JNK activation and ß-catenin nuclear aggregation.

In order to investigate the regulatory relationship, Rac1 inhibitor NSC23766 was applied in this experiment. After 24 hours of NSC23766 treatment and PDGF-BB stimulation, active Rac1 expression, pi-JNK expression and ß-catenin expression in the nucleus were detected in 5 min, 15 min and 60 min respectively; the results showed that: after PDGF-BB stimulation for appropriate time, Rac1 activity expression of pi-JNK and ß-catenin in the nucleus increased significantly. However, after the administration of different concentrations of NSC23766, all the three showed a concentration gradient-dependent decrease. This indicated that Rac1 activity played a key role in the regulation of PDGF-BB-induced JNK activation and aggregation of ß-catenin in the nucleus. To further confirm this regulatory role, Rac1siRNA interference was applied to the experiment; after giving Rac1 interference for 48 h, total Rac1 and its activity was significantly decreased, meanwhile PDGF-BB-induced pi-JNK and ß-catenin aggregation in the nucleus significantly decreased. This further illustrated

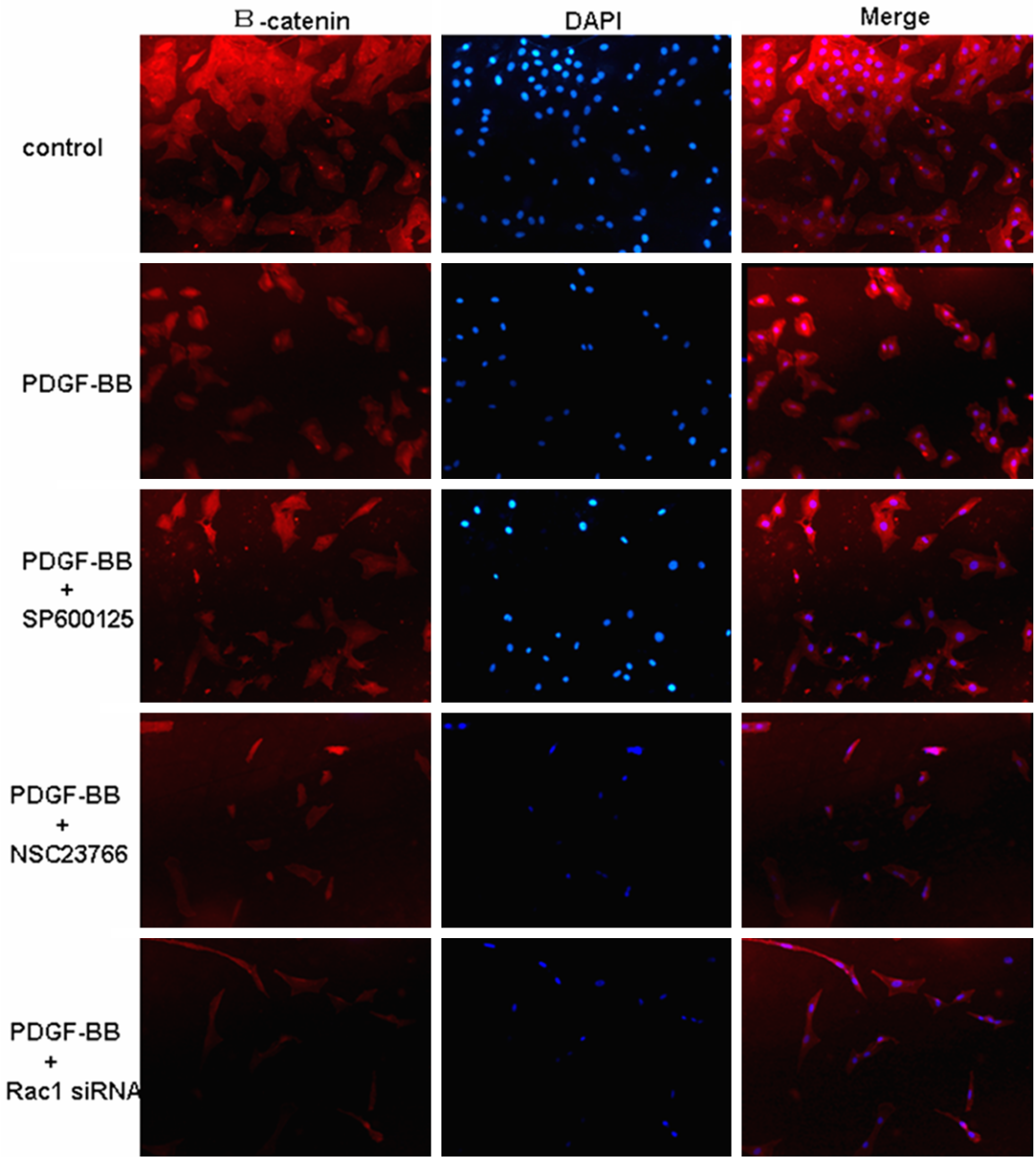


**Figure 10.** Immunofluorescence for the effect of NSC23766 (100 μmol/L), JNK inhibitor SP600125 (40 μg/L) and Rac1siRNA (50 nmol/L) on PDGF-BB-induced β-catenin nuclear accumulation (×200).

that Rac1 activity played a key role in the regulation of PDGF-BB-induced JNK activation and aggregation of ß-catenin in the nucleus; it also suggested that Rac1 may achieve the regulatory effect by JNK [11, 12].

The full name of JNK is c-Jun N-terminal kinase, playing an important role in the regulation of proliferation and migration of tumors and smooth muscle cells. In tumors, JNK induces the phosphorylation of ß-catenin and promotes them transferring into the nucleus, and thereby affect cell proliferation and migration [13]. Whether JNK plays such a regulatory role in PDGF-BB-induced Rac1 activation and ß-catenin aggregation of in the nucleus? To verify the existence of such a regulation, JNK inhibitor SP600125 was applied in experiments: After 24 hours of SP600125 treatment and PDGF-BB stimulation, active Rac1 expression, pi-JNK

expression and ß-catenin expression in the nucleus were detected in 5 min, 15 min and 60 min respectively; the results showed that: after PDGF-BB stimulation for appropriate time, Rac1 activity, expression of pi-JNK and ß-catenin expression in the nucleus increased significantly. However, after the administration of different concentrations of NSC23766, all the three showed a concentration gradient-dependent decrease. This indicated that the interaction between Rac1 and JNK co-regulated the PDGF-BB-induced nuclear aggregation of ß-catenin. Immunofluorescence test results also showed that: after PDGF-BB stimulation for 60 min, ß-catenin gathered obviously in the nucleus, but after the treatment of NSC23766, Rac1siRNA and SP600125, PDGF-BB-induced ß-catenin aggregation in the nucleus was inhibited significantly.

JNK and Rac1 are the downstream signals of PDGFR [11, 12]; vivo and vitro experiments have confirmed that PDGF is a strong stimulating factor for vascular smooth muscle cell proliferation. In carotid artery injury model, blocking PDGFR, JNK and ß-catenin could mitigate restenosis [11, 12]. Ohkawara H et al reported that rabbit atherosclerosis models, Rac1 activity in atherosclerotic plaques was increased, and it was proportional to the degree of atherosclerosis [13]. This suggested that Rac1, JNK and ß-catenin may play an important regulatory role in PDGF-BB-induced vascular smooth muscle cell proliferation and migration. While the previous reports indicated that Rac1 and JNK played a key role in regulating the classical wnt/ß-catenin signals [8]. The results of our study suggested that: PDGF activated Rac1 and JNK, and there was an interaction between Rac1 and JNK; the activity of Rac1 and JNK played a key role in PDGF-BB-induced nuclear ß-catenin accumulation. Rac1 activated JNK and JNK promoted the phosphorylation of ß-catenin residues Ser191 and Ser605, thereby regulating the internal and external nuclear distribution of ß-catenin [8].

In summary, PDGF-BB activated Rac1 and JNK in vascular smooth muscle cells, while Rac1 interacted with JNK to co-regulate the internal and external nuclear distribution of ß-catenin, thereby affecting the proliferation and migration of vascular smooth muscle cells. Further in-depth study of its regulatory mechanism can explore possible molecular therapeutic targets for restenosis and atherosclerosis.

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## Disclosure of conflict of interest

None.

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