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Netrin-1 induces the migration of Schwann cells via p38 MAPK and PI3K-Akt signaling pathway mediated by the UNC5B receptor

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ABSTRACT

Schwann cells (SCs) play an essentially supportive role in the regeneration of injured peripheral nerve system (PNS). As Netrin-1 is crucial for the normal development of nervous system (NS) and can direct the process of damaged PNS regeneration, our study was designed to determine the role of Netrin-1 in RSC96 Schwann cells (an immortalized rat Schwann cell line) proliferation and migration. Our studies demonstrated that Netrin-1 had no effect on RSC96 cells proliferation, while significantly promoted RSC96 cells migration. The Netrin-1-induced RSC96 cells migration was significantly attenuated by inhibition of p38 and PI3K through pretreatment with SB203580 and LY294002 respectively, but not inhibition of MEK1/2 and JNK by U0126-EtOH and SP600125 individually. Treatment with Netrin-1 enhanced the phosphorylation of p38 and Akt. QRT-PCR indicated that Netrin-1 and only its receptors Unc5a, Unc5b and Neogenin were expressed in RSC96 cells, among which Unc5b expressed the most. And UNC5B protein was significantly increased after stimulated by Netrin-1. In conclusion, we show here that Netrin-1-enhanced SCs migration is mediated by activating p38 MAPK and PI3K-Akt signal cascades via receptor UNC5B, which suggests that Netrin-1 could serve as a new therapeutic strategy and has potential application value for PNS regeneration.

1. Introduction

Different from central nerve system (CNS), damaged PNS is able to regenerate, which is mainly due to SCs, the main glial cells of PNS [1]. SCs develop from neural crest cells, during which process SCs transit from immature phenotype into the mature myelin-forming SCs [2]. After Peripheral nerve injury (PNI), myelinating SCs dedifferentiate into an immature state—the activated SCs, which re-enter cell cycle, synergize with macrophages removing corrupted axon and myelin debris, secrete more growth factors, and migrate into injury site forming Büngner band—a bridge guiding regenerating axons through injured site [3]. After that, denervated SCs redifferentiate to mature phenotype and remyelinate regenerating axons. However, this repair process takes a long time and the function of reinnervating targets is always hardly to fully recover, even leading to some important functions lost permanently. Thereby, to elucidate mechanisms involved in SCs migration, whereby activates SCs migration endogenously will have great potential in treating PNI.

Netrin-1, a secreted protein with highly conserved structure and function, was originally identified as one of the axonal guidance molecules during development and regeneration of nervous system [4]. Netrin-1 has a dual role in directing axon pathfinding and neuronal migration, depending on its various receptors interacted with, among which the chemoattractive role was commonly mediated by DCC receptor family (DCC and Neogenin) [5], whereas chemorepulsive role was mainly through UNC5 receptor family (UNC5A, UNC5B, UNC5C and UNC5D) cooperating with or without DCC [5,6]. In addition, Netrin-1 also plays various important roles outside of NS, such as regulating tumorigenesis, morphogenesis and angiogenesis [5,7,8].

Recently Netrin-1 and its receptors have been shown to be involved in the process of injured PNS regeneration. After PNI, Netrin-1 expression distal to nerve injured site increased significantly, mainly located in SCs [9]. Moreover, UNC5B was also

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increased, more prominent in proximal stump of injured site. Netrin-1 induced RT4 cells (a schwannoma cell line) proliferation via UNC5B [10]. Moreover, the function restore of injured PNS was much worse in Netrin-$1^{+/-}$ and Unc5b$^{+/-}$ heterozygous mice than that of the wild control mice [11,12]. However, another study reported that UNC5B decreased at injured site after PNI. And Knockdown of Unc5b at regenerating nerve front in vivo locally enhanced injured PNS regeneration significantly [13].

From the study results above we can see that there are controversies about the role played by Netrin-1 and its receptors in PNS regeneration, and little is known about the effect of Netrin-1 on SCs migration. We here found that Netrin-1 promoted SCs migration significantly in a dose-dependent manner, which was mediated by p38 MAPK and PI3K-Akt signal pathway. And UNC5B is the predominant receptor expressed in SCs.

2. Materials and methods

2.1. Reagents and antibodies

MEK1/2 inhibitor U0126-EtOH, JNK inhibitor SP600125, p38 inhibitor SB203580 and PI3K inhibitor LY294002 were purchased from Selleckchem (Houston, TX, USA). Phospho-specific or total antibodies for p38, Akt were obtained from Cell Signaling Technology (Beverly, MA, USA). UNC5B and β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and S-100 was from Abcam (Cambridge, UK).

2.2. RSC96 cells culture and identification

RSC96 SCs were obtained from Cell Bank, Chinese Academy of Sciences (Shanghai, China), which were cultured in DMEM containing 10% fetal bovine serum, 100 U/mL penicillin/streptomycin and 4 mM glutamine, within a humidified condition containing 5% CO_2 at 37 °C. Cultured RSC96 cells were identified using immunofluorescence staining of S-100, a marker of SCs, as described by previous study [14].

2.3. Cell proliferation assay

The effect of Netrin-1 on RSC96 cells proliferation was performed using Cell counting kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) assay according to the manufacturer's instructions. Briefly, RSC96 cells were seeded into 96-well plates (1×10^4 cells/ml) in 100 μL medium containing various concentrations of Netrin-1 (recombinant mouse Netrin-1, R&D system, Minneapolis, USA), ranging from 0 to 500 ng/ml. After 1, 2 and 3 days of incubation, 10 μL CCK-8 was added into each well and continued to incubate for another 4 h. Then absorbance was measured at 450 nm using a microplate fluorometer.

2.4. Cell migration assay

Cell migration assay was tested using Transwell migration chambers (8-μm pore size, Corning, NY, USA). The bottom chambers were added into different concentrations of Netrin-1 ranging from 0 to 500 ng/ml in 600 μl medium. RSC96 cells (1×10^5/ml) were seeded into top chambers at a volume of 100 μl. After incubation in 37 °C and 5% CO_2 condition for 12 h, non-migrated cells on the top chambers were removed by a cotton swab, and migrated cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. The number of stained cells was counted at nine random-selected fields per membrane. Experiments were performed in triplicate.

2.5. Western blot analysis

SCs were lysed in RIPA lysis buffer (Boster, Wuhan, China) and the quantification of whole cell lysates was performed using BCA Protein Quantitation Kit (Boster, Wuhan, China). Equal amounts of protein extracts from each group were subjected to SDS-PAGE, and then transferred to nitrocellulose membranes. After blocking with 5% skim milk, the membranes were incubated with primary antibodies against UNC5B (1:500), p38 (1:1000), p-p38 (1:1000), Akt (1:1000), p-Akt (1:1000), β-actin (1:1000), followed by the appropriate HRP-conjugated secondary antibodies. Blotted bands were visualized with ECL solution (Boster, Wuhan, China) and exposed on films.

2.6. Quantitative real-time (QRT)-PCR

Total RNA was isolated from cultured RSC96 cells using Trizol reagent (Invitrogen, USA) following the manufacturer's protocol, which was then reverse-transcribed to cDNA using ReverTra Ace qPCR RT Kit (Toyobo Co. Ltd., Osaka, Japan). Expression levels of Netrin-1, Neogenin, Dcc, Unc5a, Unc5b, Unc5c and Unc5d were assessed by QRT-PCR in RSC96 cells. Primer sequences were as follows: β-actin, F 5′-AGATCCTGACCGAGCGTGGC-3′ and R 5′-CCA GGG AGG AAG AGG ATG CG-3; Netrin-1, F 5′-AAGCAGGGCA-CAAGTCGTAT-3′ and R 5′-TGCTCTTGTCTGCCACGATG-3′; Neogenin, F 5′-AAGGAGACGAGGGACTCTAC 3 and R 5′-TCATAGAA-GACGGTCGCATC-3′; Dcc, F 5′-CAGTGGAGTCTACCGATGCT-3′ and R 5′-GGATAGCCAGAAACACAACA-3′; Unc5a, F 5′-CATCAAGCCCAG-CAAAGCAG-3′ and R 5′-CGAAGTCCTCAGCCTCAGAG-3′, Unc5b, F 5′-GTTTCCACCCCGTCAACTTC-3′ and R 5′-GGATCTTGTCGGCAGAGTCC-3′; Unc5c, F 5′-AGACCCACCTGAGCCATTAC-3′ and R 5′- GTTTCATC-TACCCTCTCGTC-3′; Unc5d, F 5′-TGTGAACATCTTCGCATCCG-3′ and R 5′-CAAGCCCGAGTACAAAGCAA-3′. QRT-PCR was performed using SYBR® Green Realtime PCR Master Mix (Toyobo Co. Ltd., Osaka, Japan). Relative mRNA levels of Netrin-1 and its receptor in RSC96 cells with β-actin used as an internal control gene were analyzed according to a comparative method with the equation expressed as $2^{-\Delta\Delta Ct}$.

2.7. Statistical analysis

Data were presented as mean ± standard deviation (SD), which was obtained from at least three independent experiments. Statistical analysis was conducted by One-way ANOVA with Bonferroni multiple comparison test followed; $p < 0.05$ was regarded to be statistically significant.

3. Results

3.1. Cellular morphology and identification of RSC96 Schwann cells

RSC96 cells cultured exhibited a spindle-like or bipolar shape, with thin and long processes on the two spindle poles of the cells, and had oval or round nuclei. All of these RSC96 cells expressed S100, a specific marker for SCs (Fig. 1A).

3.2. Netrin-1 has no effects on RSC96 cells proliferation

The effect of Netrin-1 on RSC96 cells viability was performed by CCK-8 assay. The results revealed that, as time extended, the number of SCs in all different treating-groups increased gradually. However, following 1, 2 and 3 days treatment, the average number of viable cells showed no significant difference between all Netrin-1-treating groups and blank control group (Fig. 1B). These data

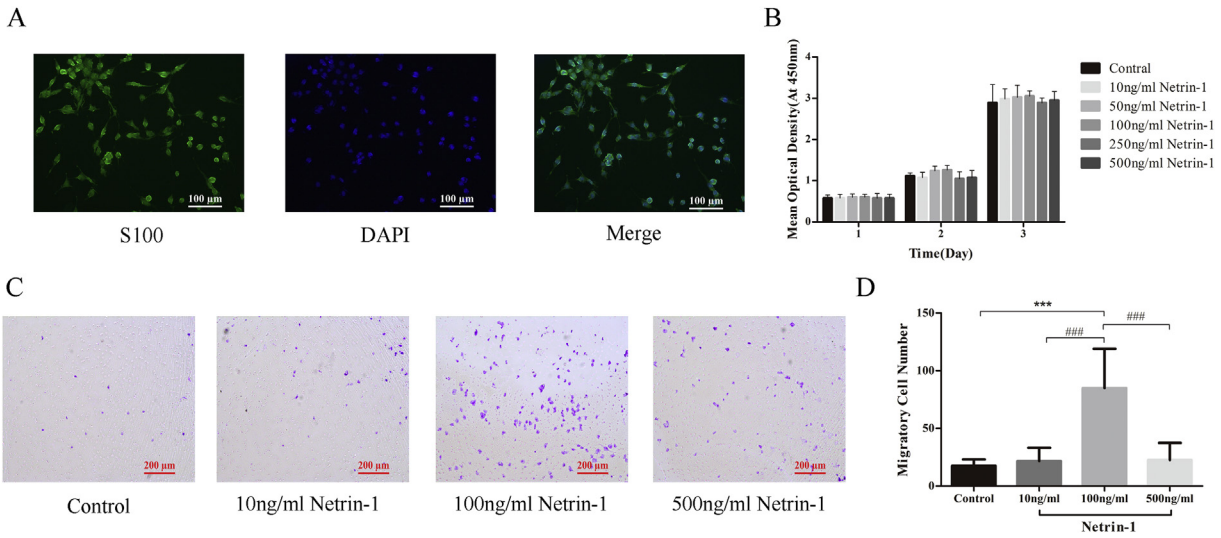

Fig. 1. (A) Fluorescent immunocytochemistry of cultured RSC96 cells stained by S-100, with nuclei counterstained with DAPI. (B) Effects of Netrin-1 on RSC96 cells proliferation performed by CCK-8 assay. (C) Migratory cells stained with crystal violet 12 h after stimulated by Netrin-1 or not in Transwell migration assay. (D) Quantitative analysis showing the effect of various concentrations of Netrin-1 on RSC96 cells migrating. Data presented as mean ± SD, n = 3, $^{***}p < 0.001$ compared with control group, $^{\#\#\#}p < 0.001$ compared with 100 ng/ml Netrin-1-treating group. One-way ANOVA.

indicated that Netrin-1 has no significant influence on RSC96 cells proliferation.

3.3. Netrin-1 induces RSC96 cells migration

The role of Netrin-1 in RSC96 cells motility was assessed by Transwell migration assay. After 12 h, the migratory cells were stained with 0.5% crystal violet (Fig. 1C). As shown in Fig. 1D, the number of migrated cells into lower chamber in groups receiving 100 ng/ml Netrin-1, but not other density of Netrin-1, was significantly higher than that of the control group. These results revealed that Netrin-1 enhanced SCs migratory activity in a dose-depended manner, promoting SCs migration within a certain concentration range, inhibiting SCs migration at a higher concentration, represented as a bell-shaped curve.

3.4. Enhanced RSC96 cells migration by Netrin-1 is p38 MAPK and PI3K-Akt signaling dependent

We next investigated whether MAPK and PI3K-Akt signal cascades participate in Netrin-1 enhanced RSC96 cells motility. RSC96 cells pretreated with SB203580(10 μM), a p38 MAPK inhibitor, for 1 h were allowed to migration for 20 h in Transwell migration assay as described in Materials and methods. The results showed that the migration of SCs promoted by Netrin-1 was significantly blocked (Fig. 2A), statistical differences existing as compared with Netrin-1 alone treating group (Fig. 2E). And similar phenomenon occurred when the inhibitor was changed to LY294002 (10 μM), a specific inhibitor against PI3k (Fig. 2B and F). However, pretreatment with JNK and MEK1/2 signaling blocker, SP600125 (10 μM) (Fig. 2C and G) and U0126-EtOH (10 μM) (Fig. 2D and H) respectively, had no observable influence on Netrin-1-induced RSC96 cells migration. These results demonstrated that p38 MAPK and PI3K-Akt signaling may be involved in enhanced SCs migration exerted by Netrin-1.

3.5. Netrin-1 increases the phosphorylation of p38 and Akt in RSC96 cells dose-dependently

Western-blot analysis was used to evaluate change of p38 MAPK and PI3K-Akt signaling activity during process of RSC96 cells migration stimulated by Netrin-1. As shown in Fig. 3A, Netrin-1 could increase the phosphorylation of p38 (p-p38) in RSC96 cells dose-dependently, with the maximal phosphorylation induced by 100 ng/ml Netrin-1. However, when Netrin-1 concentration increased to 500 ng/ml, p-p38 decreased to a level weaker than that of 100 ng/ml. And the phosphorylation of Akt (p-Akt) showed similar change (Fig. 3B). However, the levels of total p38 and Akt showed no significant difference across the different treating groups. These data revealed that Netrin-1 could activate p38 and PI3K-Akt signaling pathway in SCs.

3.6. Netrin-1 supports and protects activation of P38 MAPK and PI3K-Akt signaling cascades

As demonstrated in Fig. 3C and D, treatment with SB203580, a p38 inhibitor, or LY294002, a PI3K inhibitor, in RSC96 cells decreased expression of p-p38 or p-Akt respectively with or without 100 ng/ml Netrin-1. However, level of p-p38 or p-Akt in groups co-treated with Netrin-1 and inhibitors was still higher than that of incubation within inhibitors alone. And the expression of total p38 and Akt remained unchangeable across the different treating groups respectively. These data suggested that Netrin-1 protected or restored the activation level of p38 MAPK and PI3K-Akt signaling cascades blocked by SB203580 or LY294002 in SCs.

3.7. The efficiency of Netrin-1 on RSC96 cells might be mediated by UNC5B

To determine which receptor that may mediate Netrin-1-promoting effect on RSC96 cells migration, we investigated the expression of Netrin-1 and its receptors in RSC96 cells. The spinal cords of rats were treated as positive control. The results analyzed by QRT-PCR showed that Netrin-1 and its receptors Unc5a, Unc5b and Neogenin were expressed in RSC96 cells, while Dcc, Unc5c and Unc5d were undetectable (Fig. 4A–B). To determine which receptor expressed the most in RSC96 cells, the relative level of each receptor in RSC96 cells was expressed as the ratio indicated as the cycle threshold values of each receptor compared to that of Netrin-1 within RSC96 cells. As shown in Fig. 4C, the expression of Unc5b was much higher than that of Neogenin and Unc5a. The existing of

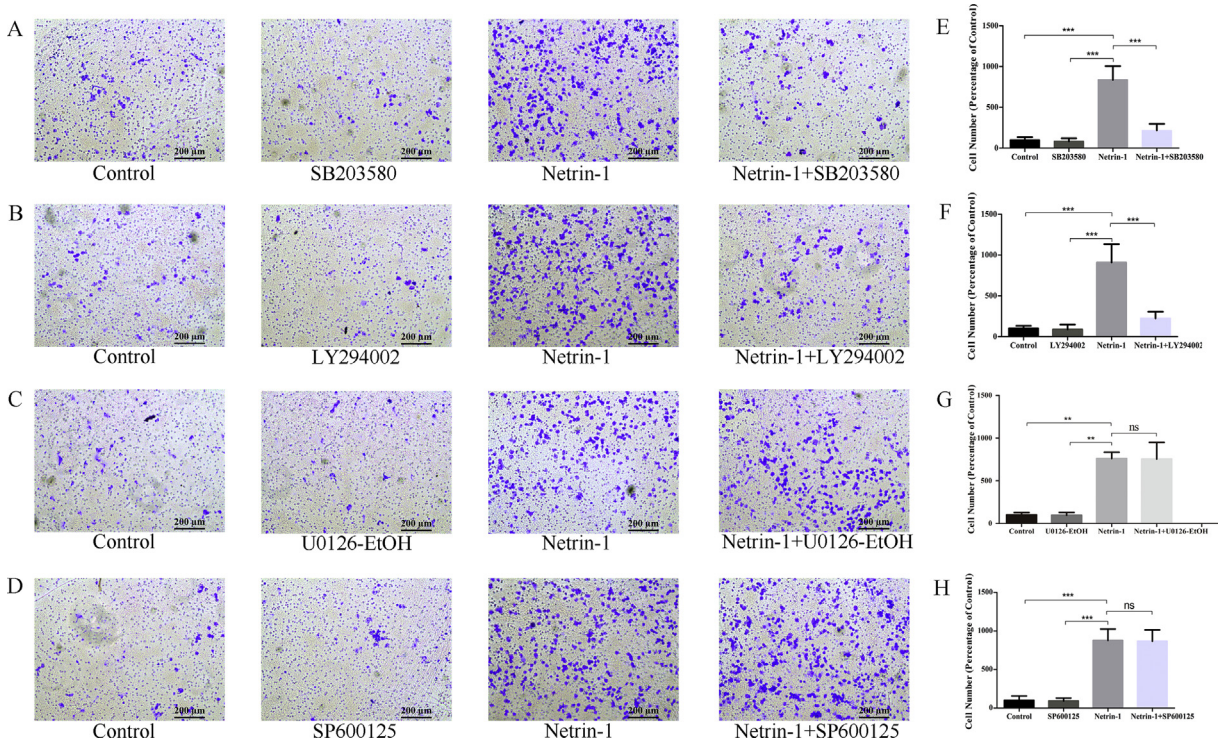

Fig. 2. The effect of p38 inhibitor SB203580 (A), PI3K inhibitor LY294002 (B), Erk1/2 inhibitor U0126-EtOH (C) and JNK inhibitor SP600125 (D), on the migration of RSC96 cells induced by 100 ng/ml Netrin-1 assessed by Transwell migration assay and subsequent quantitative analysis of the role of SB203580 (E), LY294002 (F), U0126-EtOH (G) and SP600125 (H) respectively. Data were presented as mean ± SD of the ratio of migratory cells compared to the control group, n = 3, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with Netrin-1 only treating group, ns means no significant statistical differences. One-way ANOVA.

UNC5B in RSC96 cells was further confirmed by Western-blot (Fig. 4D), and the level of UNC5B increased to a higher level following Netrin-1 stimulation (Fig. 4E). These data indicated that UNC5B may be the major receptor mediated Netrin-1-enhanced SCs movement.

4. Discussion

As SCs migration exerts a crucial role in PNS regeneration [3], to better understand molecular mechanism regulating SCs migration will greatly help us with PNI treatment. Thus we demonstrate for

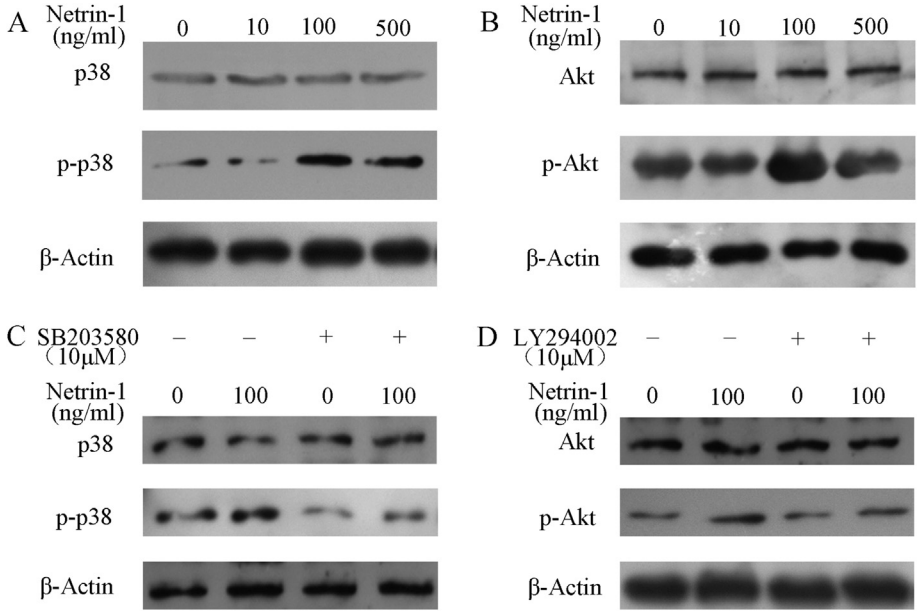

Fig. 3. Immunoblotting showing the level of phosphorylated p38 (A) and Akt (B) in RSC96 cells incubated within 100 ng/ml Netrin-1. The role of Netrin-1 in sustainment of the level of phosphorylated p38 and Akt in RSC96 cells inhibited by corresponding inhibitor SB203580 (C) and LY294002 (D).

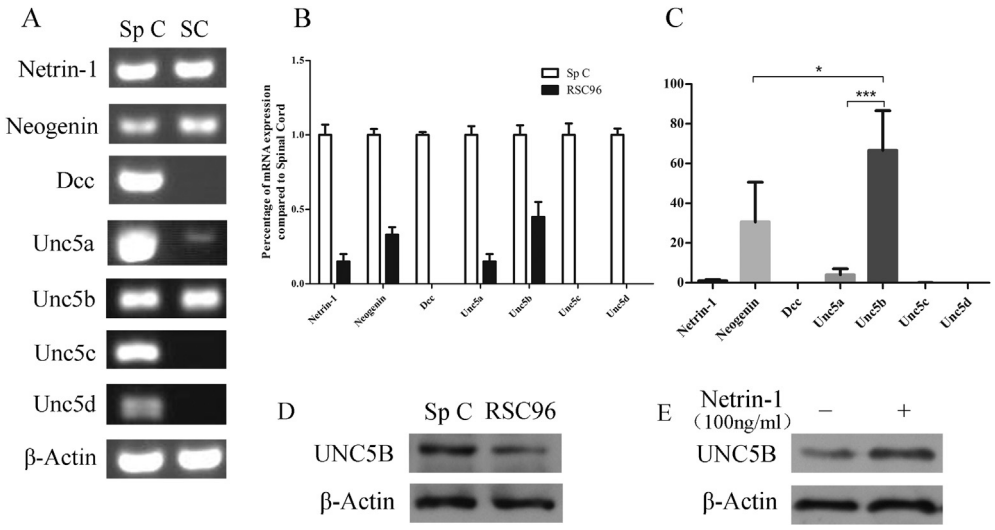

Fig. 4. (A and B) Expression of Netrin-1 and its receptor mRNA in RSC96 cells analyzed by QRT-PCR relative to that of β-actin, with rat spinal cord being as positive control. (C) Histogram showing the relative amount of each receptor mRNA in RSC96 cells normalized to that of β-actin and expressed as the ratio of each receptor compared to Netrin-1. Immunoblotting showing UNC5B protein expression in RSC96 cells (D) and change of UNC5B protein expression in RSC 96 cells with or without Netrin-1 treatment (E). Data presented as mean ± SD, n = 3, $^{*}p < 0.05$ and $^{***}p < 0.001$ compared with the relative amount of Unc5b mRNA, One-way ANOVA.

the first time that Netrin-1 could induce SCs migration via UNC5B-mediated p38 MAPK and PI3K-Akt signal pathway.

RSC96 SCs, a rat SCs line characterized by immortalization and dedifferentiation, has served as an ideal substitution for primary cultured SCs to study SCs migration [15–17]. We here found that Netrin-1 attracted RSC96 cells migration in a dose-dependent manner, with the maximal effect at 100 ng/ml concentration, which is consistent with previous study results that physiological concentration of Netrin-1 ranges from 50 to 150 ng/ml [8]. However, a higher Netrin-1 density at 500 ng/ml showed a significantly decreased migration-promoting effect on SCs, implying that as Netrin-1 concentration increases, the effect of Netrin-1 on SCs migration may gradually converts from promoting to inhibiting or from chemoattractive towards chemorepulsive. Perhaps during PNS repair, activated SCs located in injured nerve site secrete and form a Netrin-1 concentration gradient, which attracts other SCs towards injured site through UNC5B. With SCs approach injured site, the gradually higher density of Netrin-1 gradually exerts a migration-inhibiting effect on SCs to reach a balance that finally makes SCs cluster in injured site appropriately. However, the receptor and subsequent signaling mediating this migratory-inhibiting effect should be further investigated. This process is similar with the phenomenon that during axon pathfinding, as axon draws near to innervated targets, Netrin-1 increases while DCC down-regulates, leading to growth cone away from enrichment of Netrin-1 [18].

Accumulating evidences demonstrate that p38 MAPK plays an essential role in SCs motility [15,19]. Under the stimulation of Netrin-1, p38 was activated to mediate Chemotropic Turning of retinal growth cones [20]. PI3K-Akt signaling pathway also participates in the migration of many types of cells and is involved in SCs survival [21,22]. It also plays crucial role in Netrin-1 induced chemoattractive turning of Xenopus Spinal Neurons [23]. In our vitro study, pretreatment with p38 inhibitors SB203580, or PI3K inhibitors LY294002, both almost blocked migratory-enhanced effect of 100 ng/ml Netrin-1 on RSC96 cells. And under stimulation of 100 ng/ml Netrin-1, the phosphorylation of p38 and Akt was increased enormously. Therefore we believe that a certain amount of Netrin-1 attracts SCs migration through p38 and PI3K-Akt signaling. As both of the signal pathway inhibiting could almost block Netrin-1′ effect on SCs movement totally, we suppose that p38 and PI3K-Akt signal pathway must have a cooperation or integration to mediate the effect of Netrin-1 on SCs movement, instead of action by oneself independently.

Moreover, we showed that UNC5B was the predominant receptor expressed in RSC96 SCs. Upon exposed to 100 ng/ml Netrin-1, UNC5B protein expression increased significantly, which we believe that was induced by and to adapt to a higher Netrin-1 level. And Netrin-1 itself was also expressed by SCs. Perhaps Netrin-1 acts on SCs cellular activity in an autocrine and paracrine manner. UNC5B generally functions as repulsive receptors of Netrins [7]. Commissural neurons co-expressing UNC5B and DCC travel away from Netrin-1 [6]. Netrin-1 binding to UNC5B inhibits leukocytes migration [24]. However, recent studies show that UNC5B also mediates chemoattractive turning of some cell types towards Netrin-1, such as proximal tubular epithelial cells [25]. And Larrivée et al. reported that Netrin-1 by binding with UNC5B exerted repulsive effects on endothelial cell migration, thus inhibiting angiogenesis [26]. Whereas another study indicates that UNC5B promotes angiogenesis, as Unc5b knockdown mice showed decreasing of developmental placental angiogenesis [27]. Thereby, UNC5B very likely has a dual effect on cell migration, depending on ligand types and the concentration of a certain ligand, or even in a more sophisticated manner. And we here found that UNC5B mediates the chemoattractive effect of Netrin-1 at 100 ng/ml on SCs migration. Knockdown of Unc5b in SCs to verify this conclusion need to be further performed.

Our research showed that Netrin-1/UNC5B enhanced SCs migration, implying Netrin-1 may have a beneficial effect on injured PNS regeneration, which is consist with study conclusions of Madison et al. and Lee et al. that Netrin-1 and UNC5B have a beneficial role in facilitating PNS regeneration [9,10]. However, our conclusions are not in accordance with that of Webber et al.: UNC5B in vivo inhibits injured PNS regeneration promoted by SCs [13]. We suppose that these discrepancies may result from the following reasons: (1) Upon PNI, regenerated axons and activated SCs undergo dynamic changes, biological activities of which are not constant. Netrin-1 in surrounding condition and its receptors presented on SCs membrane vary at different stages. And our study only represents a certain stages of PNS regeneration and so do

others: The detection of Netrin-1, UNC5B and DCC in vivo in study of Webber et al. was all carried on within seven days following PNI, while researches of Madison et al. and Lee et al. lasted for two weeks. (2) It is well known that with Netrin-1 absence, UNC5B alone could act as the dependence receptor that initiates a cell apoptotic program [28]. Thereby inhibiting PNS regeneration by UNC5B may due to its dependence receptor role within one week after PNI in Webber et al.' study, but increasing Netrin-1 within subsequent weeks in Madison et al. and Lee et al.' study could convert inhibiting effect of UNC5B. (3) Webber et al. showed that Netrin-1 level had no significantly change before and after PNI within seven days and exogenous addition of Netrin-1 into regeneration microenvironment had no additional effect on injured PNS regeneration. Perhaps there are other ligands also activated by injury around regenerative microenvironments, which inhibit PNS regeneration via UNC5B presented on SCs or growth cones. And such ligands may antagonize positive role of Netrin-1 interaction with UNC5B. The dynamic change of expression and role of Netrin-1 and its receptors in PNS regeneration in vivo should be further clarified.

In summary, our findings demonstrate a positive role of Netrin-1 in SCs migration via UNC5B-mediated p38 MAPK and PI3-Akt signaling pathway, which suggests that Netrin-1/UNC5B system is very likely to become a novel therapeutic target for clinical application, appropriately utilized of which could promote injured PNS regeneration significantly.

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Transparency document

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