The microRNAs let-7 and miR-9 down-regulate the axon-guidance genes Ntn1 and Dcc during peripheral nerve regeneration

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Critical. Neurons extend axons such that they reach their targets precisely by navigating through a complex regeneration microenvironment with an extreme precision process known as axon guidance during nerve repair and regeneration (4, 5). It is essential for axons to target their destinations precisely for functional recovery.

In recent years, microRNAs (miRNAs)3 are a class of small noncoding RNAs that have emerged, as key post-transcriptional regulators in a majority of the eukaryotic cells (6, 7). In animals, miRNAs bind to partially complementary sites in mRNAs, leading to translational repression and mRNA deadenylation and degradation (8–10). Previously, we identified a series of differentially expressed miRNAs and found that these miRNAs affect the microenvironment after peripheral nerve regeneration (11–15). Debris removal, axonal growth, and axon guidance are key elements that affect the regenerative microenvironment and contribute to peripheral nerve regeneration. Our previous studies showed that after sciatric nerve injury, let-7 and miR-1 regulate the phenotype of SCs by directly targeting the nerve growth factors, NGF and BDNF separately, and further promoting the axon growth of sciatic nerve (12, 13). In addition, we found that miR-340 could target and regulate the tPA for debris clearance and axon growth (14). In the current study, we explored the regulation mechanism of axon guidance during peripheral nerve regeneration.

Ntn1 attracts and repels the motor axons in distinct populations (19). Deleted in colorectal carcinoma (Dcc) is one of the major receptors of Ntn1 (20). Ntn1 binds to Dcc, mediates its axon guidance, and guides the axonal growth and directional cell migration (21–23). It has also been demon-

Peripheral nerve injury is common in clinical cases and affects the patients’ quality of life severely (1–3). Thus, investigating the molecular mechanisms underlying the peripheral nerve regeneration for the development of medical therapies is critical. Neurons extend axons such that, they reach their targets precisely by navigating through a complex regeneration microenvironment with an extreme precision process known as axon guidance during nerve repair and regeneration (4, 5). It is essential for axons to target their destinations precisely for functional recovery.

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3 The abbreviations used are: miRNA, microRNA; SC, Schwann cell; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; Ntn1, Netrin-1; Dcc, deleted in colorectal carcinoma; qRT, quantitative real-time; DMEM, Dulbecco’s modified Eagle’s medium; DRG, dorsal root ganglion; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; tPA, tissue plasminogen activator.
stratified that the expression levels of Ntn1 and Dcc increased significantly during sciatic nerve autoimmune inflammation. The elevated levels of Ntn1 and Dcc promote cell survival and axon regeneration (24). These studies imply that Ntn1 and Dcc might play critical roles in promoting axon growth and guiding axons to their final destinations during peripheral nerve regeneration.

Following peripheral nerve injury, the expression levels of several genes are altered and these genes affect the biological behaviors of neurons and SCs (5, 25–27). Emerging studies have showed that miRNAs are critical fine-tuning regulatory molecules in axon guidance (4, 28, 29). Because the regulatory mechanisms of Ntn1 and Dcc are yet incomprehensive, our current study aims to investigate the regulatory mechanisms and roles of miRNAs on Ntn1 and Dcc.

Results

**Ntn1 and Dcc expressions were elevated after sciatic nerve injury**

Quantitative real time-PCR (qRT-PCR) analysis showed that the mRNA expressions of Ntn1 in the sciatic nerve stumps after nerve crush was increased from day 1 after nerve injury as compared with 0 h after nerve injury, and significantly increased at days 7 and 14 after nerve injury (Fig. 1A). Western blot analysis showed that the protein expressions of Ntn1 in the crushed nerve stumps were also increased from day 1 after nerve injury as compared with the 0-h control, and significantly increased at days 4, 7, and 14 (Fig. 1B). Detailed analysis displayed that at day 4 post-nerve injury, expression of the Ntn1 protein was significantly elevated as compared with that of the 0-h control, whereas the mRNA expression did not differ significantly (Fig. 1, A and B). Also, the mRNA and protein expressions of Dcc in the crushed nerve segments were increased after nerve crush, reaching a peak value at days 7 and 14 (Fig. 1, C and D). The protein expression of Dcc reached a peak value at day 7 post-injury, whereas the mRNA expression of Dcc did not reach a peak value until day 14 post-injury (Fig. 1, C and D).

**let-7 negatively regulated Ntn1 by directly targeting its 3’-UTR**

The TargetScan prediction and the negative correlation between miRNA and mRNA expression patterns after sciatic nerve injury were demonstrated previously by microarray and Solexa sequencing (30, 31). A total of 9 miRNAs were identified as potential regulatory miRNAs of Ntn1, whereas 4 miRNAs, let-7, miR-17, miR-27, and miR-128, were conserved across species (Fig. 2A). These miRNAs were subjected to luciferase reporter assay to explore whether they could directly target Ntn1. The results suggested that only let-7 could significantly decrease the luciferase activity (Fig. 2B). In addition, WT Ntn1 or mutant 3’-UTR of the let-7-binding site was constructed and introduced into the downstream region of the luciferase gene (Fig. 2C). Co-transfection of the WT Ntn1-containing plasmid and let-7 mimic
reduced luciferase activity, whereas mutant Ntn1-containing plasmid failed to show this effect (Fig. 2D), indicating that let-7 directly bound to the 3′-UTR of Ntn1.

Furthermore, we observed the effects of let-7 on the mRNA and protein abundances of Ntn1. The results from qRT-PCR showed that neither let-7 mimic nor let-7 inhibitor affect the mRNA expression of Ntn1 (Fig. 2E). On the other hand, Western blot analysis suggested that let-7 mimic down-regulated the protein expression of Ntn1, whereas let-7 inhibitor up-regulated the protein expression of Ntn1 (Fig. 2F). Moreover, an animal model of peripheral nerve crush injury was utilized to investigate the in vivo effect of let-7 agonomer and let-7 antagonomer
Figure 3. Effects of let-7 on Ntn1 secretion. Primary SCs (A) and RSC96 SCs (B) were transfected with let-7 mimic (let-7), let-7 inhibitor (Anti-let-7), mimic control (miR Con), or inhibitor control (Anti-miR Con), respectively. The Ntn1 secretions from both primary SCs and RSC96 SCs transfected with let-7 mimic were significantly decreased, whereas the Ntn1 secretions from both primary SCs and RSC96 SCs transfected with let-7 inhibitor were increased significantly as compared with that transfected with the corresponding controls (n = 3/group). C, let-7-induced reduction of Ntn1 secretion was rescued by co-transfection with let-7 mimic plus Ntn1 3′-UTR plasmid (n = 3/group). *, p < 0.05; **, p < 0.01 versus control.

on expression of the Ntn1 protein. As compared with the corresponding control group, the application of let-7 agomir significantly decreased expression of the Ntn1 protein, whereas no significant difference was detected in the application of let-7 antagonist (Fig. 2G). Both in vitro and in vivo results suggested that let-7 targeted and regulated the expression of Ntn1. Furthermore, as previously obtained qRT-PCR results demonstrated, members of the let-7 family were consistently up-regulated at day 1 and down-regulated at day 7 post-nerve as compared with the 0-h control (12). Interestingly, compared with the temporal expressions of Ntn1 (Fig. 1A), the expression of let-7 was correlated with that of Ntn1.

let-7 inhibited the secretion of Ntn1 from SCs

To identify the impact of let-7 on cellular function, primary SCs and RSC96 SCs were transfected with let-7 mimic, let-7 inhibitor, or nontargeting negative controls. Because Ntn1 is a secreted protein, ELISA was used to determine the effects of let-7 on the secretion of Ntn1. In the cultured primary SCs, transfection with the let-7 mimic and let-7 inhibitor significantly decreased and increased the secretion of Ntn1, respectively, as compared with the control (Fig. 3A). Similarly, in RSC96 SCs, let-7 mimic and let-7 inhibitor suppressed and elevated the secretion of Ntn1, respectively (Fig. 3B). Subsequently, RSC96 SCs were co-transfected with let-7 mimic and Ntn1 3′-UTR plasmid. Consistent with these observations in Fig. 3B, let-7 mimic induced a reduction in Ntn1 secretion. However, this effect was abolished by Ntn1 3′-UTR plasmid (Fig. 3C), indicating that the inhibitory effect of let-7 on Ntn1 secretion was mediated by targeting Ntn1.

miR-9 negatively regulated Dcc by directly targeting its 3′-UTR

The potential regulatory miRNAs of Dcc were also investigated. Similar to the identification of regulatory miRNAs of Ntn1, candidate miRNAs of Dcc were discovered using TargetScan prediction software and the correlation between the expression of miRNAs and mRNAs. Among these identified miRNAs, miR-9, miR-27, miR-128, miR-192, miR-203, and miR-489 were highly conserved across species and hence, selected for subsequent studies (Fig. 4A). After construction and insertion of miR-9, miR-27, miR-128, miR-192, miR-203, or miR-489 and the 3′-UTR of Dcc into the downstream region of the luciferase reporter gene, miR-9 significantly reduced luciferase activity (Fig. 4B). Moreover, after the construction and insertion of miR-9 and the WT or mutant 3′-UTR of Dcc into the downstream region of the luciferase reporter gene (Fig. 4C), only the plasmid containing the WT 3′-UTR of Dcc led to a robust reduction in luciferase activity (Fig. 4D). qRT-PCR and Western blot analysis showed that miR-9 failed to affect the mRNA expression of Dcc (Fig. 4E) but distinctly affected the protein expression (Fig. 4, F and G). Furthermore, the in vivo effect of miR-9 was explored in a rat model of sciatic nerve crush. Western blot analysis suggested that miR-9 agomir and miR-9 antagonist suppressed and elevated the protein expression of Dcc, respectively (Fig. 4, H and I). Also, the expression pattern of miR-9 after sciatic nerve crush was negatively correlated with that of Dcc (Fig. 4F). These results demonstrated that miR-9 directly bound to Dcc and regulated its expression.

miR-9 and Dcc affected SC migration

Transwell migration assay was conducted to observe the effect of miR-9 and Dcc on modulation of the phenotype of cultured primary SCs. Compared with the SCs transfected with nontargeting negative control, transfection with miR-9 mimic or miR-9 inhibitor induced a significant decrease or increase in the rate of cell migration, respectively (Fig. 5A). Then, the SCs were transfected with Dcc siRNA that stably reduced Dcc expression (Fig. 5, B and C). Similar to the effect of miR-9 mimic, Dcc siRNA reduced the migration rate of SCs (Fig. 5D). Moreover, co-transfection of Dcc siRNA with the miR-9 inhibitor abrogated the effect of the miR-9 inhibitor partially, indicating that miR-9 affects the migration of SCs by targeting Dcc (Fig. 5E).

let-7 and miR-9 inhibit the expression of Ntn1 and Dcc in dorsal root ganglia neurons (DRGs) and suppress the axon outgrowth

In addition to SCs, we also tested whether let-7 and miR-9 could target Ntn1 and Dcc in neuron cells by transfecting the mimic or inhibitor of let-7 and miR-9 in the DRGs. Western blotting assay showed that let-7 and miR-9 mimics inhibited the expression of Ntn1 and Dcc proteins, whereas let-7 and miR-9 inhibitors promoted the expression of the proteins (Fig. 6, A
Figure 4. Identification of potential regulatory miRNAs targeting Dcc. A, cross-species conservation of binding to target Dcc 3'-UTR for miR-9, miR-27, miR-128, miR-203, miR-489, and miR-192. B, relative luciferase activities of 293T cells transfected with miR-9 mimic (miR-9), miR-27 mimic (miR-27), miR-128 mimic (miR-128), miR-203 mimic (miR-203), miR-489 mimic (miR-489), and mimic control (miR Con) (n = 3/group). C, sketches of the construction of WT or mutant p-Luc-UTR vectors for miR-9. D, the relative luciferase activities of 293T cells transfected with WT or mutant p-Luc-UTR and miR-9 mimic (miR-9) or mimic control (miR Con) (n = 3/group). E, the mRNA expressions of Dcc in SCs transfected with miR-9 mimic (miR-9) and mimic control (miR Con) or with miR-9 inhibitor (Anti-miR-9) and inhibitor control (Anti-miR Con) (n = 3/group). F and G, the protein expressions of Dcc in SCs transfected with miR-9 mimic (miR-9) and mimic control (miR Con) or with miR-9 inhibitor (Anti-miR-9) and inhibitor control (Anti-miR Con) (n = 3/group). H and I, the protein expressions of Dcc in injury sciatic nerve stumps was due to receiving an injection of a mixture containing miR-9 agomir (miR-9) and agomir control (miR Con) or with miR-9 antagonor (Anti-miR-9) and antagonor control (Anti-miR Con), respectively (n = 3/group). J, the temporal expression pattern of miR-9 in the injured nerve stumps after sciatic nerve injury (n = 3/group). *, p < 0.05 and **, p < 0.01 versus control.
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and B). Immunohistochemistry showed that after transfection with let-7 or miR-9 mimics, axon outgrowth was remarkably reduced in DRGs. Conversely, axon outgrowth was enhanced in DRGs after transfecting with let-7 and miR-9 inhibitor (Fig. 6, C and D).

A collaborative regulatory network of Ntn1 and Dcc

A previous study showed that Src activation triggers NF-κB activation, which in turn, directly activates Lin28 transcription and rapidly reduces the let-7 expression (32). miR-9 was regarded as a negative regulator of Lin28 (33). Also, Dcc deletion severely reduces the Src activation (34, 35). These results suggested a putative regulatory pathway between NF-κB, Lin28, Ntn1, Dcc, and Src and implied that let-7 and miR-9 might be involved in this regulatory pathway. To test this hypothesis, we transfected SCs with let-7 and found that let-7 elevated the expression of miR-9 and inhibited the expression of Ntn1, Dcc, Src, and NF-κB proteins (Fig. 7A). Similarly, transfection with Lin28 siRNA elevated the expressions of let-7 and miR-9 genes and reduced the expression of Ntn1, Dcc, Src, and NF-κB proteins (Fig. 7B). These preliminary results showed that miR-9 and let-7 target Dcc and Ntn1, respectively, and are also linked with Src, NF-κB, and Lin28.

Discussion

Peripheral nerves exhibited a specific ability to regrow after injury. However, patients with severe peripheral nerve injury often showed poor nerve regeneration and incomplete functional recovery (36). The failure of functional recovery might be attributed to the termination of the growth of axons that do extend accurately into the corresponding effector tissues. The accurate pathfinding of the growing axons is guided by diffusible or bound factors that attract or repel the axonal growth cones (37). Ntn1 and its receptor Dcc play essential roles in axon guidance. The current study investigated the expression patterns of Ntn1 and Dcc, and found that the expressions were altered after sciatic nerve injury. Also, Ntn1 and Dcc might be involved in peripheral nerve injury and regeneration.

miRNAs are critical post-transcriptional regulators that participate in axonal navigation in the central nervous system (4, 38–40), the proliferation and migration of SCs, and the axonal growth of neurons in the peripheral nervous system (11–14). Furthermore, this study showed that miRNAs might also regulate axon guidance in the peripheral nervous system. Target prediction algorithm and conservative analysis suggested that Ntn1 and Dcc might be potential target genes of let-7 and miR-9 miRNAs. The data from the luciferase reporter assay confirmed that let-7 and miR-9 directly targeted the 3’-UTR of Ntn1 and Dcc, respectively. Also, we observed that let-7 and miR-9 regulated the Ntn1 and Dcc expression independently in vitro and in vivo, as well as that Ntn1 and Dcc were real target genes of let-7 and miR-9, respectively.

Specifically, Ntn1 protein is secreted into the extracellular environment and plays a guiding role in axon guidance after nerve injury. The current study showed that let-7 affected the amount of secreted Ntn1 from SCs, and thus, affect axon guidance via binding of Ntn1 to its receptor Dcc in the axons. Therefore, we tested the effect of miR-9 on the expressions of Dcc in DRGs. Western blotting results indicated that miR-9 altered the expression of Dcc in DRGs, indicating that miR-9 regulated axon guidance by targeting Dcc in DRGs.

Axon outgrowth and axon guidance are two critical aspects in nerve development and regeneration with indivisible correlation, therefore, we observed the influence of let-7 and miR-9 on the axon outgrowth of DRGs. The results showed that the let-7 and miR-9 mimics decreased the axon outgrowth, whereas it was increased by let-7 and miR-9 inhibitors. These results were consistent with those described in a previous study (40–43).

Previously, we reported that after sciatic nerve injury, differentially expressed let-7 and miR-1 regulated the SC phenotype, such as proliferation and migration by targeting NGF and BDNF independently and further influenced the axon growth of sciatic nerve (12, 13). Interestingly, miR-340 targets and regulates the tPA such as to affect the debris clearance and the axon growth (14). The current study identified that let-7 and miR-9 were regulatory miRNAs of Ntn1 and Dcc, and therefore the addition of let-7 and miR-9 to a regulatory network ensured that damaged axons proliferated in the correct direction. Also, we systematically explored the miRNA-mediated regulation of axon growth (let-7 and miR-1), debris clearance (miR-340), and axon guidance (let-7 and miR-9) by the corresponding targets NGF, BDNF, tPA, Ntn1, and Dcc. Some studies reported that Ntn1, Dcc, Src, NF-κB, and Lin28 formed a complex regulatory pathway (32–35, 44). Combined with our previous and current works, a regulation schematic diagram can be summarized in Fig. 8. It clearly clarifies the regulation pathway with Lin28, let-7, and miR-9 as the core regulation center (Fig. 8).

Debris removal, axonal growth, and axon guidance are three key elements that contribute to peripheral nerve regeneration. Following nerve injury, SCs, undergoing dedifferentiation/proliferation and migration help to improve the regenerative microenvironment by the removal of myelin debris, secretion of several factors, and formation of Bungner band (45, 46). The injured axon is triggered by intrinsic regenerative abilities. The current results showed that these miRNAs in the regulatory network controlled the regenerative microenvironment of the injured nerves and affected further nerve regeneration. These findings would aid in understanding the complex regulatory mechanisms during peripheral nerve injury and regeneration. However, additional comprehensive studies are essential for an in-depth perspective on these molecular mediators, coupled with the related bioprocesses and signaling pathways, are orchestrated to activate the intrinsic regenerative programs of peripheral nerves. Thus, the present study not only provides an insight into miRNA regulation of peripheral nerve regeneration but also opens a novel therapeutic avenue for the repair of peripheral nerve injury by regulating the production of Ntn1 and Dcc.

Experimental procedures

Animal surgery and tissue preparation

Sprague-Dawley rats were purchased from the Experimental Animal Center of Nantong University, Nantong, Jiangsu,
China. All experimental and animal handling procedures were executed according to the Institutional Animal Care Guidelines of Nantong University and all animal experiments were ethically approved by the Administration Committee of Experimental Animal, Jiangsu, China.

Adult, male Sprague-Dawley rats (180–220 g) were anesthetized with intraperitoneal injection of complex narcotics (85 mg/kg of trichloroacetaldehyde monohydrate, 42 mg/kg of magnesium sulfate, and 17 mg/kg of sodium pentobarbital). Rat sciatic nerve was exposed through an incision on the lateral aspect of the mid-thigh of the left hind limb. A 3-mm long segment of sciatic nerve was crushed two times (15 s each time with a 3-s interval) using a pair of hemostatic forceps. To minimize the discomfort and possible painful mechanical stimulation, the rats were housed in large cages with sawdust bedding post-surgery. The crushed sciatic nerve with both nerve ends

Figure 5. Effect of miR-9 and Dcc on SC migration. A, images showing that primary SCs migrated to the bottom of the Transwell chamber after transfection with miR-9 mimic (miR-9), miR Con, miR-9 inhibitor (Anti-miR-9), and anti-miR Con, respectively. Dot plots showing the cell migration ability (normalized to control) of transfected SCs, n = 3/group, scale bar = 100 μm. B, the Dcc protein expression, and C, the Dcc mRNA expression in primary SCs were decreased by transfection with Dcc siRNA (si DCC), respectively (n = 3/group). D, the migration ability of primary SCs was decreased by Dcc siRNA (si DCC), n = 3/group, scale bar = 100 μm. E, an increase in the cell migration of SCs transfected with miR-9 inhibitor (Anti-miR-9) was partially rescued by cotransfection with Dcc siRNA (si DCC) (n = 3/group, scale bar = 100 μm). *, p < 0.05 and **, p < 0.01 versus control.
(1-mm long) was harvested at 0 h and on days 1, 4, 7, and 14 after nerve crush.

For in vivo experiments, the crush site was injected with a mixture (a volume ratio of 1:1) of Matrigel (BD Biosciences, Billerica, MA) and miRNA agomir, miRNA antagomir, or the corresponding controls (Ribobio, Guangzhou, China), respectively. On day 3 post-surgery, the sciatic nerve segments together with both nerve ends (1-mm long) were collected for subsequent Western blot analysis to determine the protein expression levels of Ntn1 and Dcc.

**Cell culture and transfection**

Primary SCs were collected from the sciatic nerves of 1-day-old postnatal Sprague-Dawley rats and further isolated from the fibroblasts using anti-Thy1.1 antibody and rabbit complement (Sigma) as described previously (12). The purity of SCs was assessed by immunostaining with anti-NF200 showing that axon outgrowth was decreased in DRGs, which was transfected with let-7 mimic (Anti-let-7) and miR-9 mimic (Anti-miR-9). On the other hand, the axon outgrowth was increased in DRGs, which was transfected with let-7 inhibitor (Anti-let-7) and miR-9 inhibitor (Anti-miR-9). Scale bar = 50 μm. D, the average length of axon outgrowth in DRGs (n = 3/group). *, p < 0.05 versus control.
DRGs were removed from 1-day-old postnatal Sprague-Dawley rats and digested in 1% collagenase type I (Sigma) for 30 min at 37 °C, and 0.25% trypsin (Invitrogen) for 10 min, followed by mechanically triturated using a pipette to obtain single cell suspension. Subsequently, the cells were centrifuged at 900 rpm for 10 min on 15% BSA (Sigma) in PBS (Invitrogen) to remove the SCs. Purified DRGs were cultured on poly-L-lysine–coated glass plates in Neurobasal (Invitrogen) and B-27 (Invitrogen). Cultured cells were transfected with miRNA mimics, miRNA inhibitors, or the corresponding controls (Ribobio) using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer’s instructions.

qRT-PCR

Total RNA was extracted using TRIzol (Life Technologies) according to the manufacturer’s instructions. The contaminating DNA was removed using RNeasy spin columns (Qiagen, Valencia, CA). The quality of the isolated RNA samples was evaluated using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) and the quantity of RNA samples was determined using a NanoDrop ND-1000 spectrophotometer (Infiringen Biotechnology Inc., City of Industry, CA). An equivalent of 20 ng of RNA samples was reversely transcribed using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and stem-loop RT primers (Ribobio) according to the manufacturer’s instructions to determine the miRNA expression. The mRNA was reverse transcribed into cDNA using a Prime-Script Reagent Kit (TaKaRa, Dalian, China) according to the manufacturer’s instructions. qRT-PCR was performed using SYBR Green Premix Ex Taq (TaKaRa) with Ntn1 and Dcc primers on an Applied Biosystems StepOne real-time PCR system to evaluate the mRNA expression of the corresponding genes. The sequences of primers were as follows (5’–3’): Ntn1, forward, GCAGCATGGAAGAACCTGA and reverse, CGGCCTATACGACTTGTTG; Dcc, forward, ACAACAGGAGCAGGCTTT and reverse, GCAGATACAGCGTGCCAGGGTT; Gapdh, forward, CCTTCATTGACCTCAACTCATG and reverse, CTTTCATCATGTGAGGAAGAC. The reactions were carried out in triplicate. The relative expressions of miRNAs and mRNAs were calculated using the comparative \(2^{-\Delta\Delta Ct} \) method; U6 and Gapdh served as the reference genes, respectively.
Western blot analysis
Protein extracts were prepared from nerve tissues. Equivalent amounts of isolated protein were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Then, these membranes were blocked with 5% nonfat dry milk in Tris-HCl–buffered saline (TBS) at room temperature and probed with Ntn1 (1:1000, Abcam, Cambridge, MA) and Dcc (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies, followed by horseradish peroxidase-conjugated second antibody. Subsequently, the membrane was developed with enhanced chemiluminescence reagent (Cell Signaling, Beverly, MA) and exposed to Kodak X-Omat Blue film (PerkinElmer Life Sciences, Boston, MA). The immunoreactive bands were analyzed quantitatively using Grab-it 2.5 and Gelwork software.

Cell migration assay
The migration of SCs was examined using 6.5-mm Transwell chambers with 8-μm pores (Costar, Cambridge, MA). The bottom surface of each membrane was coated with 10 μg/ml of fibronectin. An equivalent of 106 cells/ml of SCs in 100 μl of DMEM was transferred to the top chambers of each Transwell and allowed to migrate at 37 °C in a 5% CO2 incubator. A volume of 600 μl of complete medium was injected into the lower chambers. The upper surface of each membrane was cleaned with a cotton swab at the indicated time points. The cells adhering to the bottom surface of each membrane were stained with 0.1% crystal violet, imaged, and enumerated using a DMR inverted microscope (Leica Microsystems, Bensheim, Germany). The assays were repeated three times in triplicate.

Plasmid construction and luciferase assay
The luciferase reporter vector was constructed by transferring the fragment of luciferase from pGL3-Basic vector using HindIII and XbaI and inserting a multiple cloning sequence after the XbaI site in the pcDNA3 vector. The 3'-UTR of Ntn1 or Dcc was amplified from the genomic DNA by PCR and subcloned into the region directly downstream of the stop codon in the luciferase gene in the luciferase reporter vector to generate p-Luc-UTR reporter plasmid. Then, overlap PCR was used to construct the 3'-UTR mutant reporter plasmid. The primers used to generate the WT, and mutant 3'-UTRs were as follows (5’–3’): Ntn1 3'-UTR, forward, TCCGGTACCCCAGGCCCTGGAGAAATGA and reverse, GGTTCTAGACAAGGTtTCTCAAGAGACAAG; Ntn1 3’-UTR mutant, forward, GCGCCG-
TGACCAAGCTATGTCTTTGTCATTT and reverse, GAC-ATAGCTGGTCACGGGCCTCTTCCAG; Dec 3’-UTR, forward, CGGAATTCATTGATGGAACACCTGGTAGG and reverse, CCGCTCGAGGCCAACATATTGGAACAA-AAG; Dec 3’-UTR mutant, forward, GAAACATC-CATCCTTCCAGCTATGGAG and reverse, ACTCTCAC-ACCTCATTAGCTGAAAGATTG. The sequences of WT and mutant 3’-UTR were confirmed by sequencing.

For luciferase assay, HEK 293T cells were seeded in 24-well plates and transfected with a mixture of 120 ng of p-Luc-UTR, 20 pmol of miRNA mimic, and 20 ng of Renilla luciferase vector pRL-CMV (Promega, Madison, WI) using the Lipofectamine 2000 transfection system (Invitrogen). The luciferase activity was measured from the cell lysate at 24 h post-transfection using the Dual-Luciferase Reporter Assay System according to the recommended protocols (Promega).

ELISA

Primary SCs and RSC96 SCs were transfected with 10 nm let-7 mimic, 40 nm let-7 inhibitor, and the corresponding dose negative controls, respectively, using Lipofectamine RNAiMAX transfection reagent (Invitrogen). For reverse analysis, RSC96 SCs were transfected using the Lipofectamine 2000 transfection system (Invitrogen) with miRNA mimic control and vector control plasmid, let-7 mimic and vector control plasmid, or let-7 mimic and Ntn1 3′-UTR plasmid. Subsequently, primers used to amplify the Ntn1 3′-UTR fragment were as follows (5′–3′): forward, TCCGGTACCCCAGGCCCAACCTGGAGAAAATG; reverse, TCCGGTACCTAAACCCTAAATACAGTG. Then, the Ntn1 3′-UTR fragment was cloned into the pcDNA3 to construct the Ntn1 3′-UTR plasmid, whereas the empty pcDNA3 vector served as the control. An equivalent of 6 μg of plasmid was added in a well of 6-well plate, whereas the concentration of let-7 mimic was 10 nm. The fetal bovine serum-free DMEM was replaced, and the reaction incubated for an additional 48 h. Finally, the medium was collected and passed through a 0.22-μm filter (Millipore) to obtain the supernatant that was concentrated to an appropriate volume by freeze-drying. The protein levels of Ntn1 were measured using a Ntn1 enzyme-linked immunosorbent assay (ELISA) kit (Molecular Innovations, Novi, MI). The data from 3 independent cultures, each comprising of triplicate wells, were collected and expressed in triplicate wells were collected and expressed as mean ± S.E.

Immunohistochemistry

DRGs were fixed in 4% paraformaldehyde after culturing for 48 h and immunostained with mouse anti-neurofilament 200 (1:500, Sigma) primary antibody, followed by the fluorescent-labeled secondary antibodies (1:400, Invitrogen). Images were acquired by fluorescence microscopy (Leica). The experiment was repeated three times.

Statistical analysis

The Student’s t test or analysis of variance was used for statistical analysis conducted on SPSS 15.0 software (SPSS, Chicago, IL). p < 0.05 was considered statistically significant. All data were expressed as mean ± S.E.
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