

Novel microRNA, miR-sc6, modulates Schwann cell phenotype via targeting ErbB4

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Abstract. MicroRNAs (miRNAs) are non-coding RNAs that regulate various tissues and organs, including the nervous system. Peripheral nerve injury is a common pathology of the nervous system and leads to differential expressions of a variety of miRNAs. Previously, a group of novel miRNAs have been identified in rat proximal nerve segments after sciatic nerve transection. However, the biological functions of these novel miRNAs remain undetermined. The aim of the current study was therefore to identify the function of a novel miRNA, miR-sc6, following nerve injury. Its target genes and effects on phenotypic modulation of Schwann cells were determined using a miR-sc6 mimic transfection. These observations contribute to the understanding of miRNA involvement in peripheral nerve injury and the cognition of regulatory mechanisms in peripheral nerve regeneration.

Introduction

MicroRNAs (miRNAs) are a family of endogenous non-coding RNAs that account for >3% of all human genes (1). miRNAs, were previously considered as junk RNAs. However, previous studies indicated that they are involved in the regulation of

the stability and translation of up to 60% of coding mRNAs in humans (2,3). Through direct binding to the 3'-untranslated region (3'-UTR) of target coding mRNAs, miRNAs repress the expression of their target genes and affect a variety of essential biological processes (4-6). In addition, miRNAs play significant roles in various pathological processes including cardiovascular diseases, viral diseases, immune diseases and cancer (7-11). Emerging studies showed that miRNAs are abundant in the nervous system and are correlated with several nervous system pathologies, including neurodevelopmental abnormalities, neurodegenerative disorders and nerve injuries (7,12,13).

Nerve injury is a common clinical issue that induces nerve tissue damage and target organ dysfunction, and is generally classified into two categories: Central nerve injury and peripheral nerve injury, in accordance with the injury site. While central nerve injuries regenerate poorly, the functional recovery of peripheral nerve injuries are significantly better (14,15). A major reason for the high regeneration abilities of injured peripheral nerves is the presence of Schwann cells. Schwann cells are unique types of glial cells in the peripheral nervous system, and support neurons by forming myelin under physiological conditions (16,17). After peripheral nerve injury, Schwann cells proliferate and migrate to the injury site, engulf axon and myelin debris, and form bands of Bungner to guide the directional regrowth of axons (18). Therefore, factors that promote Schwann cell proliferation and migration may contribute to the repair and regeneration of injured peripheral nerves.

Numerous miRNAs have been identified to be differentially expressed in the proximal nerve segment after peripheral nerve injury (19,20). These differentially expressed miRNAs have been reported to modulate various phenotypic processes of Schwann cells, including apoptosis, proliferation, migration, differentiation and myelination; processes which may affect peripheral nerve regeneration (21-23). In a previous study, a total of 98 novel miRNAs have been discovered and functionally annotated in the rat sciatic nerve segment after sciatic nerve transection (24). These novel miRNAs were perfectly mapped to the rat genome, with their hairpin structures and low free energy levels obtained (24). Here, we focused on miR-sc6, a newly identified miRNA, and examined its effects

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on Schwann cell proliferation and migration, and determined the target genes of miR-sc6.

Materials and methods

Animal surgery. The present study was approved by the Administration Committee of Experimental Animals in (Jiangsu, China; no. 20170302-016). Sprague Dawley (SD) rats were obtained from the Experimental Animal Center (animal license nos. SCXK (Su) 2014-0001 and SYXK (Su) 2012-0031). A total of 30 adult male SD rats (weight, 180-220 g; age, 2 months) were randomly separated into 5 groups (at 0, 1, 4, 7 and 14 days after nerve injury) with 6 rats/group, and used for sciatic nerve transection as previously described (24). Briefly, after anesthesia, 10 mm of rat sciatic nerve was exposed, lifted, and resected at the site proximal to the division of tibial and common peroneal nerves. After the surgical incisions were closed, animals were housed in temperature- and humidity-controlled environment, maintained under a 12-h light/dark cycle, and were allowed free access to water and food. Rats were sacrificed by decapitation at 0, 1, 4, 7 and 14 days after nerve transection, and 5 mm of proximal sciatic nerve segments were collected for subsequent reverse transcription-quantitative polymerase chain reaction (RT-qPCR) experiments. A total of 60 neonatal male SD rats were used for Schwann cell isolation as previously described (25). Briefly, after anesthesia, sciatic nerve was exposed, and an 8 mm of nerve segment was collected for subsequent cell isolation.

Schwann cell culture and transfection. Primary Schwann cells were isolated from sciatic nerve segments using trypsin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Isolated cells were purified by the treatment of anti-Thy1.1 antibody (1:1,000; cat. no. M7898; Sigma-Aldrich; Merck KGaA) and rabbit complement (Sigma-Aldrich; Merck KGaA), as previously described (25). Purified Schwann cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific Inc.), 1% penicillin and streptomycin (Invitrogen; Thermo Fisher Scientific Inc.), 2 μ M forskolin (Sigma-Aldrich; Merck KGaA), and 10 ng/ml human heregulin- β 1 (HRG; Sigma; Merck KGaA). Schwann cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂, and were passaged for no more than three times prior to use. Cultured Schwann cells had a purity >98% confirmed via immunostaining with S100 β (1:200; cat. no. ab52642; Abcam, Cambridge, MA, USA), a marker of Schwann cells. For cell transfection, Schwann cells were transfected with a miR-sc6 mimic (sense, 5'-UGGGGACAGGGCAAGGUGG-3' and antisense, 5'-CCACCUUGC CCUGUCCCCCA-3'), mimic (sense, 5'-UUCUCCGAACGU GUCACGU-3' and antisense, 5'-ACGUGACACGUUCGG AGAA-3'), miR-sc6 inhibitor (sequence, 5'-CCACCUUGC CCUGUCCCCCA-3') or inhibitor control (sequence, 5'-ACG UGACACGUUCGGAGAA-3') (Guangzhou RiboBio Co., Ltd., Guangzhou, China) using Lipofectamine[®] RNAiMAX transfection reagent (Invitrogen; Thermo Fisher Scientific Inc.), according to the manufacturer's protocols. Briefly, the miR-sc6 mimic, mimic control, miR-sc6 inhibitor or inhibitor control was diluted in Opti-MEM I Medium (Gibco; Thermo

Fisher Scientific Inc.). Lipofectamine[®] RNAiMAX was then added into the corresponding diluted complexes and incubated for 10-20 min at room temperature. The transfection mixture containing the reagent and miRNA diluted in Opti-MEM was then added onto the Schwann cells at 30-50% confluence, which were then incubated at 37°C under 5% CO₂ for 24 h. The experiment was repeated three times.

5-ethynyl-2'-deoxyuridine (EdU) proliferation assay. Schwann cells were suspended and seeded at a density of 2x10⁵ cells/ml onto 96-well plates precoated with 0.01% poly-L-lysine (Sigma; Merck KGaA), and transfected with miR-sc6 mimic, mimic control, miR-sc6 inhibitor or inhibitor control for 36 h. EdU (Guangzhou RiboBio Co., Ltd., Guangzhou, China) was then added into culture media and cells were cultured for another 24 h. The cells were then fixed with 4% paraformaldehyde (PFA; Xilong Scientific, Guangzhou, China) for 30 min at room temperature, stained with Hoechst 33342 (Beyotime Institute of Biotechnology, Haimen, China) for 10 min at room temperature, and observed with a DMR fluorescence microscope (magnification, x200; Leica Microsystems GmbH, Wetzlar, Germany). The proliferation rate was calculated by dividing the number of EdU-positive cells to the number of total cells. The experiment was repeated three times.

Transwell assay. Schwann cells were transfected with miR-sc6 mimic, mimic control, miR-sc6 inhibitor or inhibitor control. Following 36 h of transfection, the cells were suspended in DMEM, and seeded at a density of 3x10⁵ cells/ml onto the top chamber of a 6.5 mm transwell insert with 8 μ m pores (Costar; Corning Incorporated; Corning, NY, USA). A total of 500 μ l DMEM supplemented with 10% FBS were added to the bottom chamber of transwell pre-coated with 10 μ g/ml fibronectin. Schwann cells left on the top chamber were wiped off by a cotton swab after a 24 h incubation at 37°C with 5% CO₂. The Schwann cells that migrated to the lower chamber were fixed with 4% paraformaldehyde for 30 min at room temperature, stained with 0.1% crystal violet for 10 min at room temperature and then observed with a DMR inverted microscope (magnification, x200; Leica Microsystems GmbH). Crystal violet staining the Schwann cells was dissolved by acetic acid. The migratory ability of Schwann cells was calculated by the optical density value of the dissolved crystal violet dye. The experiment was repeated three times.

Renilla luciferase assay. The 3'-UTR of netrin-1 (Ntn1), erbB2 receptor tyrosine-protein kinase 4 (ErbB4) or protein kinase C α (Prkc α) was amplified and subcloned into the downstream region of the luciferase gene stop codon in the luciferase reporter vector to generate the p-Luc-UTR reporter plasmid (Promega Corporation, Madison, WI, USA). A total of 120 ng constructed p-Luc-UTR reporter plasmid was combined with 20 pmol miR-sc6 mimic and 20 ng Renilla luciferase vector pRL-CMV (Promega Corporation) to generate a mixture. On the day of the assay 293 cells were seeded onto 24-well plates and then transfected with the mixture using the Lipofectamine 2000 transfection system (Invitrogen; Thermo Fisher Scientific, Inc.), and subsequently cultured for an additional 24 h. The firefly and Renilla luciferase activities were measured using the dual-luciferase reporter assay system (Promega Corporation).

RT-qPCR. RNA samples were isolated from proximal sciatic nerve segments using TRIzol (Life technologies; Thermo Fisher Scientific, Inc). Total RNA was reverse transcribed into cDNA using Omniscript® Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany). RT-qPCR experiments were conducted using an Applied Biosystems StepOne real-time PCR System and QuantiNova™ SYBR® Green PCR Kit (Qiagen GmbH). The following primer pairs were used for qPCR: ErbB4 forward, 5'-GCATGTGATGGAATCGGCAC-3' and reverse, 5'-TCC CCATGAATGCCAGTGAC-3'. The thermocycling conditions were as follows: Initial denaturation for 5 min at 95°C; 40 cycles of denaturation for 30 sec at 95°C, annealing for 45 sec at 60°C, and extension for 30 sec at 72°C; and final extension for 5 min at 72°C. The expression levels of ErbB4 were determined using the $2^{-\Delta\Delta C_q}$ method (26), and were normalized to the internal reference gene GAPDH. The relative expression levels of ErbB4 at 1, 4, 7 and 14 days after sciatic nerve transection were compared with its expression levels at 0 day.

Bioinformatics analysis. The 3'-UTRs of all genes in the rat genome were downloaded, and sequence alignment was conducted using Basic Local Alignment Search Tool (BLAST) (24). The candidate target genes of miR-sc6 were predicted using TargetScan. Functional genes associated with cell proliferation and migration were examined using Database for Annotation, Visualization, and Integrated Discovery (DAVID) (27) bioinformatic resources. The minimum free energy of hybridization between miR-sc6 and the candidate target genes was determined using the RNAhybrid software (bibiserv.cebitec.uni-bielefeld.de/rnahybrid) (28).

Statistical analysis. Statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). Three independent experiments were performed, and the results were presented as mean \pm standard error of the mean. Student's t-test or one-way analysis of variance followed by Dunnett's multiple comparisons test was used for statistical comparison. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-sc6 promotes Schwann cell proliferation. Schwann cells are the main cell types in the sciatic nerve stumps and play important roles during peripheral nerve regeneration (29), therefore, the effect of miR-sc6 expression on Schwann cell proliferation was investigated. Transfection using a miR-sc6 mimic or miR-sc6 inhibitor significantly increased or decreased the expression of miR-sc6, respectively (Fig. 1A). EdU assay was used to determine the effect of miR-sc6 upregulation/downregulation on the proliferative ability of Schwann cells. Schwann cells transfected with miR-sc6 mimic after 36 h exhibited a higher ratio of the number of EdU-positive cells to the number of total cells, compared with the negative control group (Fig. 1B). The results revealed that transfection using miR-sc6 mimics led to a significantly elevated proliferation rate (Fig. 1B). Schwann cells were also transfected with a miR-sc6 inhibitor or its corresponding negative control. Transfection with the miR-sc6 inhibitor led to a significantly reduced proliferation rate compared with control (Fig. 1C).

miR-sc6 promotes Schwann cell migration. To examine the effect of miR-sc6 on Schwann cell migration, Transwell migration assay was performed. Schwann cells transfected with miR-sc6 mimic showed an increased number of migrated cells compared with those transfected with mimic control, suggesting that an increased expression of miR-sc6 promoted the migration of Schwann cells (Fig. 2A). However, transfection with the miR-sc6 inhibitor significantly decreased the migration of Schwann cells (Fig. 2B).

Identification of potential candidate target genes of miR-sc6. Considering that miRNAs perform their biological functions through the direct regulation of their target genes (30), the candidate target genes of miR-sc6 were predicted. Bioinformatic analysis suggested that ErbB4, Ntn1 and Prkca were potential target genes of miR-sc6. The sequences of ErbB4, Ntn1 and Prkca in the seed region were also completely complementary to that of miR-sc6 (Fig. 3A). All three candidate target genes displayed negative free energy as determined by using the RNAhybrid software (Fig. 3A). The results revealed that ErbB4 has a free energy between -20 and -30 kcal/mol, indicating that ErbB4 mRNA may bind with miR-sc6.

The expression association between miR-sc6 and its potential target genes ErbB4, Ntn1 and Prkca were determined using previously obtained Solexa sequencing and microarray data (24,31). The heatmap of the temporal expression patterns of miR-sc6 revealed upregulation, while, the expression of ErbB4, Ntn1 and Prkca were downregulated after sciatic nerve transection. The results obtained on day 7 revealed that the expression pattern of miR-sc6 may be negatively associated with that of ErbB4, Ntn1 and Prkca (Fig. 3B).

ErbB4 is a candidate target gene of miR-sc6. Renilla luciferase assay was performed to investigate further whether miR-sc6 directly targets the 3'-UTR of ErbB4, Ntn1 and Prkca. Transfection using miR-sc6 mimic and p-Luc-UTR reporter plasmid containing the 3'-UTR of ErbB4 showed a significantly reduced relative luciferase activity compared with cells transfected with its corresponding non-targeting negative control (Fig. 4A). In contrast, 293 cells transfected with miR-sc6 mimic and p-Luc-UTR reporter plasmid containing the 3'-UTR of Ntn1 or Prkca did not show an altered relative luciferase activity (Fig. 4A).

The expression levels of ErbB4 in the proximal nerve segments at 0, 1, 4, 7 and 14 days after sciatic nerve transection were measured to investigate further the association between miR-sc6 and its candidate target gene ErbB4. Previous results from Solexa sequencing showed that miR-sc6 was upregulated after sciatic nerve transection, reaching a peak value at 7 days after nerve injury (24). The temporal expression patterns of ErbB4 at different timepoints after sciatic nerve transection displayed opposite trends. The expression levels of ErbB4 were decreased at 1, 4, 7 and 14 days after nerve injury, reaching the lowest value at 7 days (Fig. 4B). These results collectively suggest that ErbB4 might be the direct target of miR-sc6.

Discussion

The peripheral nerve system contains an internal regenerative capacity after traumatic injury. However, the functional

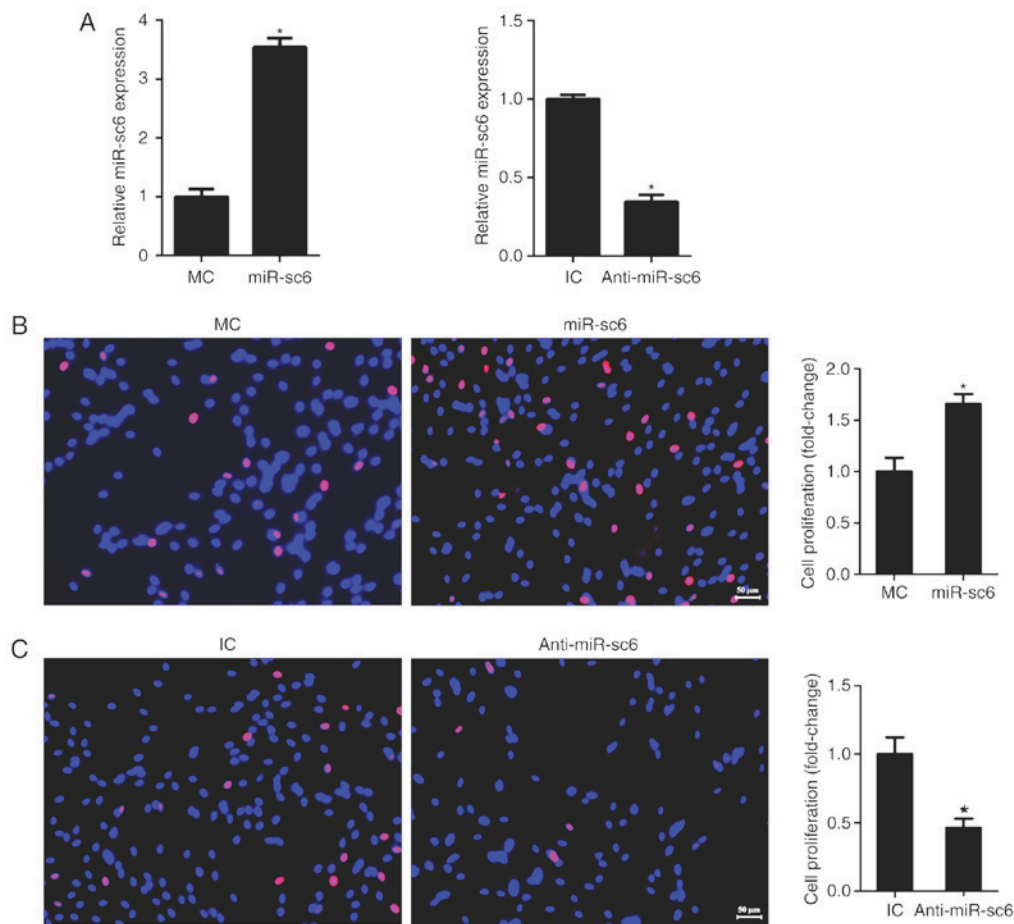


Figure 1. Effect of miR-sc6 on Schwann cell proliferation. (A) The expression of miR-sc6 was increased and decreased by transfection with miR-sc6 and anti-miR-sc6, respectively. (B) Transfection of Schwann cells with miR-sc6 increased cell proliferation as compared with that of MC. (C) Transfection of Schwann cells with anti-miR-sc6 decreased cell proliferation compared with that of IC. 5-ethynyl-2'-deoxyuridine staining is shown in red while Hoechst 33342 staining is shown in blue. Data were summarized from three independent experiments. * $P < 0.05$ vs. MC and IC. miR-sc6, miR-sc6 mimic; anti-miR-sc6, miR-sc6 inhibitor; MC, mimic control; IC, inhibitor control; miR, microRNA.

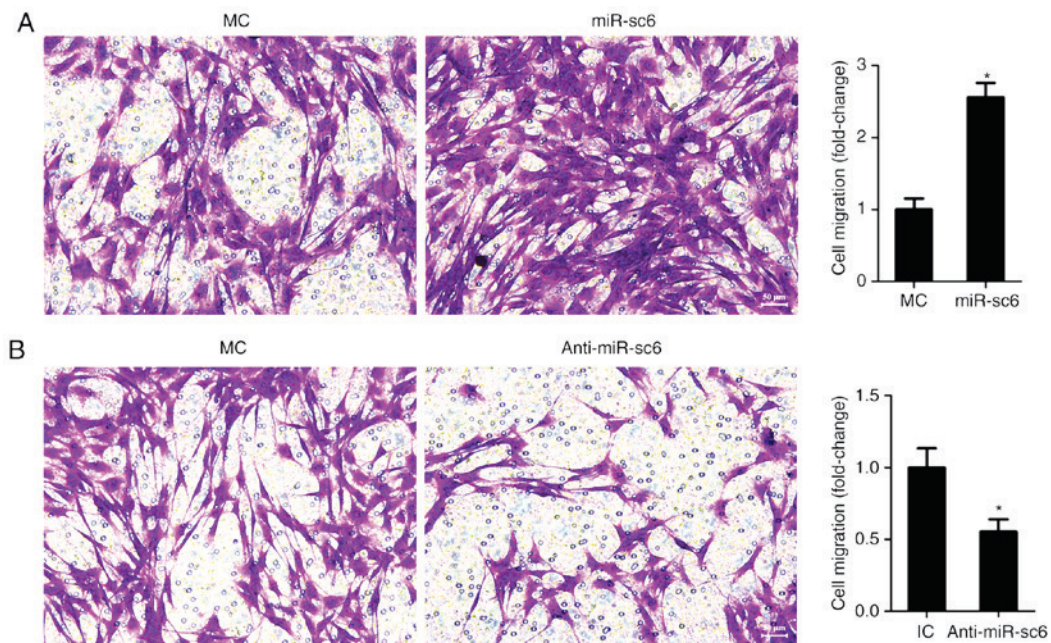


Figure 2. Effect of miR-sc6 on Schwann cell migration. (A) Transfection of Schwann cells with miR-sc6 increased cell migration compared with that of MC. (B) Transfection of Schwann cells with anti-miR-sc6 decreased cell migration compared with IC. Migrated Schwann cells are shown in purple. Data were summarized from three independent experiments. * $P < 0.05$ vs. MC and IC. miR-sc6, miR-sc6 mimic; anti-miR-sc6, miR-sc6 inhibitor; MC, mimic control; IC, inhibitor control; miR, microRNA.

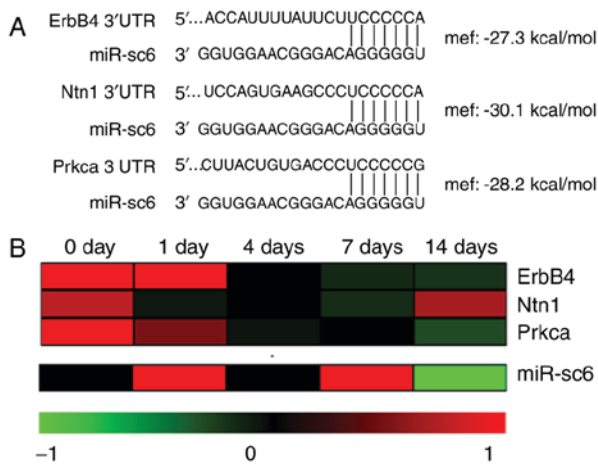


Figure 3. Identification of candidate target genes of miR-sc6. (A) List of miR-sc6 binding sites and its candidate target genes ErbB4, Ntn1 and Prkca. Predicted minimal energy values were listed. (B) Heatmap of the temporal expression patterns of miR-sc6, ErbB4, Ntn1 and Prkca in the proximal sciatic nerve segments 0, 1, 4, 7 and 14 days after peripheral nerve injury. ErbB4, erbB2 receptor tyrosine kinase 4; Ntn1, netrin-1; Prkca, Protein kinase C.

recovery of peripheral nerve injury is generally not desirable (32). Many strategies, including the applications of neurotrophic factors (nerve growth factor, brain-derived neurotrophic factor and glial-derived neurotrophic factor), Schwann cells and stem cells, have been used to facilitate peripheral nerve repair and regeneration (33-36). Almost immediately after nerve injury, Schwann cells in the distal region begin to dedifferentiate, engulf axon and myelin debris, and clear a regenerative path. At the same time, Schwann cells in the proximal region proliferate, migrate and form bands of Bungner to guide axonal regrowth (37,38). Therefore, phenotypic modulation of Schwann cells largely contributes to nerve regeneration. Recently, it has been demonstrated that miRNAs are able to regulate Schwann cell phenotype and may be of potential therapeutic interest for the treatment of peripheral nerve injury in the future (21,39,40).

The advance of high-throughput analysis, especially of gene sequencing analysis, is of benefit to the identification of differentially expressed genes following peripheral nerve injury (41). Previously, in our laboratory, sequencing was performed to discover regular alterations in the expression levels of miRNAs in proximal nerve segments after rat sciatic nerve transection (19). In addition, by using Solexa sequencing, a group of novel miRNAs were identified after rat sciatic nerve transection and, were named as miR-scs since they were discovered in rat sciatic nerve segments (24). The biological functions of certain of these novel miRNAs were further investigated, and the results showed that these miRNAs could affect the proliferation and migration of Schwann cells. For example, miR-sc3 was found to promote Schwann cell proliferation and migration by targeting astrotactin 1 (Astn1), while miR-sc4 and miR-sc8 played an inhibitory role on Schwann cell proliferation and migration by targeting cyclin-dependent kinase 5 activator 1 (Cdk5r1) and the epidermal growth factor receptor (Egfr), respectively (40,42,43).

The present study focused on miR-sc6, a miRNA verified as a novel miRNA in rat whose expression levels were

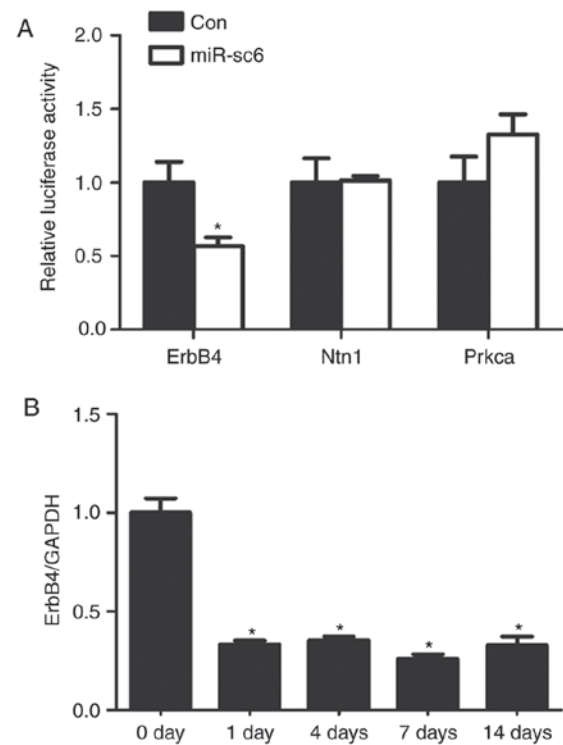


Figure 4. miR-sc6 induces inhibition of ErbB4 expression by targeting the 3'-untranslated region in 293 cells. (A) Transfection of 293 cells with p-Luc-UTR and miR-sc6 reduced the relative luciferase activity of ErbB4. Data were summarized from three independent experiments. *P<0.05 vs. con. (B) The expression levels of ErbB4 in the proximal sciatic nerve segments at 0, 1, 4, 7 and 14 days after peripheral nerve injury. Data were summarized from three independent experiments. *P<0.05 vs. 0 day. MiR-sc6, miR-sc6 mimic; miR, microRNA; ErbB4, erbB2 receptor tyrosine kinase 4; Ntn1, netrin-1; Prkca, protein kinase C; con, control.

upregulated after rat sciatic nerve transection. By using EdU proliferation assay and Transwell migration assay, it was showed that increased expression of miR-sc6 promoted Schwann cell proliferation and migration, while decreased expression of miR-sc6 suppressed Schwann cell proliferation and migration. These results suggested that peripheral nerve injury-induced upregulation of miR-sc6 may support the proliferation and migration of Schwann cells, and thus contribute to subsequent axon regrowth and nerve regeneration. Bioinformatic analysis and Renilla luciferase assay identified ErbB4 as the target gene of miR-sc6. The temporal expression patterns of ErbB4 in proximal sciatic nerve segments at 0, 1, 4, 7 and 14 days after sciatic nerve transection were also negatively associated with that of miR-sc6, implying that miR-sc6 may perform its biological functions by negatively regulating ErbB4 expression.

ErbB4 is a transmembrane receptor-tyrosine kinase that belongs to the ErbB receptor family. The activation of the neuregulin-1/ErbB signaling pathway can promote the proliferation of neoplastic Schwann cells and the mitogenesis in malignant peripheral nerve sheath tumors (44,45). A previous study examined changes in ErbBs mRNA expression during peripheral nerve regeneration and found that ErbB4 was downregulated at 7, 14 and 28 days post-injury (46). This was consistent with our observations. According to a previous study, other members of the ErbB family exhibited different expression trends [for example, ErbB1 (Egfr) was downregulated, ErbB3

was upregulated, and ErbB2 was not differentially expressed in the rat median nerve] (46). ErbB2 and ErbB3 were reported to be essential for the migration and myelination of Schwann cells in zebrafish (47). However, another study showed that ErbB2 was dispensable for the maintenance of established myelinated peripheral nerves in mice (48). Despite the complex and paradoxical roles of ErbB2, the direct biological effect of ErbB4 on Schwann cells remains unclear. Transgenic mice expressing a dominant-negative ErbB4 receptor in non-myelinating Schwann cells expressing a dominant negative ErbB4 exhibited elevated cell apoptosis and cell proliferation, which presented with a progressive peripheral neuropathy (49). The results of the present study revealed that ErbB4 (a target gene of miR-sc6) may modulate Schwann cell physiology. Further investigations will be performed to determine whether ErbB4 regulated Schwann cell proliferation and migration.

Taken together, the present study demonstrated the possible biological functions of miR-sc6, a novel miRNA that was dysregulated following peripheral nerve injury. The data may deepen our understanding of the cellular and molecular mechanisms involved in peripheral nerve injury and regeneration.

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Availability of data materials

All data generated or analysed during this study are included in this published article.

Authors' contributions

ZC and TQ conceived and designed the experiments. CC, QL, HH, XW, PW and TQ performed the experiments. CC and TQ analyzed the data. QL and TQ contributed reagents, materials and analysis tools. TQ wrote the manuscript.

Ethical approval and consent to participate

The present study was approved by the Administration Committee of Experimental Animals (Jiangsu, China; no. 20170302-016). All applicable international, national and institutional guidelines for the care and use of animals were followed.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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