RESEARCH ARTICLE

Increased levels of miR-3099 induced by peripheral nerve injury promote Schwann cell proliferation and migration

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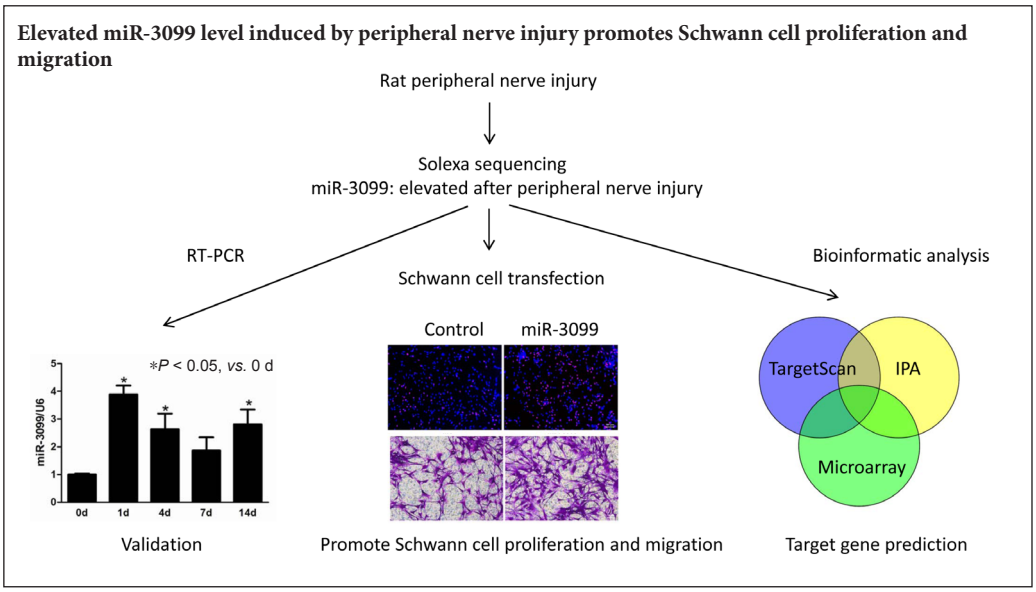
Funding: *This study was supported by the Postgraduate Research & Practice Innovation Program of Jiangsu Province of China, No. KYCX17-1910 (to QYL); and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions of China (PAPD).*

Graphical Abstract



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doi: *10.4103/1673-5374.245478*

Received: *May 23, 2018*
Accepted: *August 20, 2018*

Abstract

MicroRNAs (miRNAs) can regulate the modulation of the phenotype of Schwann cells. Numerous novel miRNAs have been discovered and identified in rat sciatic nerve segments, including miR-3099. In the current study, miR-3099 expression levels following peripheral nerve injury were measured in the proximal stumps of rat sciatic nerves after surgical crush. Real-time reverse transcription-polymerase chain reaction was used to determine miR-3099 expression in the crushed nerve segment at 0, 1, 4, 7, and 14 days post sciatic nerve injury, which was consistent with Solexa sequencing outcomes. Expression of miR-3099 was up-regulated following peripheral nerve injury. EdU and transwell chamber assays were used to observe the effect of miR-3099 on Schwann cell proliferation and migration. The results showed that increased miR-3099 expression promoted the proliferation and migration of Schwann cells. However, reduced miR-3099 expression suppressed the proliferation and migration of Schwann cells. The potential target genes of miR-3099 were also investigated by bioinformatic tools and high-throughput outcomes. miR-3099 targets genes Aqp4, St8sia2, Tnfsf15, and Zbtb16 and affects the proliferation and migration of Schwann cells. This study examined the levels of miR-3099 at different time points following peripheral nerve injury. Our results confirmed that increased miR-3099 level induced by peripheral nerve injury can promote the proliferation and migration of Schwann cells.

Key Words: *nerve regeneration; peripheral nerve injury; miR-3099; sciatic nerve; gene expression; Schwann cells; proliferation; migration; target genes; mechanisms; neural regeneration*

Chinese Library Classification No. *R453; R319; Q2*

Introduction

The peripheral nervous system, unlike the central nervous system, has some capacity to regenerate (Gu et al., 2011, 2014). One important reason for the regenerative ability of the peripheral nervous system is that it possesses a particular type of glial cells, Schwann cells (Dezawa and Adachi-Usami, 2000; Jessen and Mirsky, 2005; Chen et al., 2007; Castelnovo et al., 2017). Under physiological conditions, Schwann cells are in contact with axons and provide nutrition and physical support to axons to the benefit of neuronal survival (Griffin and Thompson, 2008; Monk et al., 2015; Boerboom et al., 2017; Wong et al., 2017). Myelinating Schwann cells

wrap around axons, forming a myelin sheath, and contribute to the integrity and function of axons (Lee and Wolfe, 2000; Salzer et al., 2008; Salzer, 2015). Following peripheral nerve injury, Schwann cells dedifferentiate, proliferate and migrate to the injured site (Xiang et al., 2017). There they help to construct a permissive microenvironment for subsequent axon regrowth and nerve regeneration (Bunge, 1993; Frostick et al., 1998; Stoll and Muller, 1999; Zhang et al., 2016). Considering the critical roles played by Schwann cells, treatments that are able to accelerate the proliferation and migration of Schwann cells may help to promote the repair and regeneration of injured peripheral nerves.

Emerging studies showed that not only coding genes but also some non-coding genes, such as microRNAs (miRNAs), could affect the phenotype of Schwann cells (Yu et al., 2012b, 2015; Ghibaudi et al., 2017; Li et al., 2017a; Yi et al., 2017; Qian et al., 2018; Wang et al., 2018). miRNAs are a group of endogenous, evolutionarily conserved non-coding RNA molecules that regulate numerous complex biological activities by binding to and suppressing their target genes (Ambros, 2004; Bartel, 2004; Krol et al., 2010). Our previous studies revealed that many miRNAs could modulate Schwann cell proliferation and migration; miR-182 could inhibit the proliferation and migration of Schwann cells by targeting FGF9 and NTM, respectively (Yu et al., 2012a); miR-1 could inhibit the proliferation and migration of Schwann cells by targeting BDNF (Yi et al., 2016a); let-7 could inhibit the proliferation and migration of Schwann cells by targeting nerve growth factor (Li et al., 2015); miR-340 could inhibit the migration of Schwann cells by targeting tPA (Li et al., 2017b); and miR-132 could promote the migration of Schwann cells by targeting PRKAG3 (Yao et al., 2016).

Using sequencing analysis, a series of novel miRNAs were identified in rat sciatic nerve segments following peripheral nerve injury (Li et al., 2011). Detailed studies of these novel miRNAs suggested that miR-sc3 and miR-sc8 could promote and inhibit the proliferation and migration of Schwann cells, respectively (Gu et al., 2015; Yi et al., 2016b). The biological functions of other novel miRNAs remain largely undetermined. In this study we focused on miR-3099, a miRNA that previously had not been identified in rats, and studied its regulatory effect on Schwann cell phenotype modulation and the potential target genes of miR-3099.

Materials and Methods

Animals

Adult and neonatal Sprague-Dawley rats were purchased from the Experimental Animal Center of Nantong University, China (license No. SCXK [Su] 2014-0001 and SYXK [Su] 2012-0031). Thirty adult male Sprague-Dawley rats, weighing 180–220 g, were subjected to surgical crush of sciatic nerves. Sixty neonatal 1-day-old Sprague-Dawley rats of either sex were used to collect primary Schwann cells.

All the experimental procedures were approved by the Administration Committee of Experimental Animals of Nantong University, China (license number of The Tab of Animal Experimental Ethical Inspection: 20170302-016).

Sciatic nerve injury rat modeling

The adult male Sprague-Dawley rats were subjected to surgical crush of sciatic nerves as previously described (Li et al., 2017). Briefly, after anesthesia with intravenous 4% chloral hydrate, rat sciatic nerve segments were exposed and crushed with a forceps three times, each for 10 seconds. Proximal sciatic nerve stumps were collected at 0, 1, 4, 7, and 14 days following sciatic nerve injury.

Real-time reverse transcription-polymerase chain reaction

Sciatic nerve stumps from 2 rats per group were mixed and used for each biological replication. Total RNAs were extracted from these nerve segments using TRIzol (Life technologies, Carlsbad, CA, USA) and reverse transcribed using Omniscript Reverse Transcription Kit (Qiagen, Valencia, CA, USA). Real-time Real-time reverse transcription-polymerase chain reaction (RT-PCR) was conducted using QuantiNova SYBR Green PCR Kit (Qiagen) on an Applied Biosystems StepOne real-time PCR system. Primers were obtained from RiboBio, Guangzhou, China. The relative quantification of miR-3099 was performed using the $2^{-\Delta\Delta Ct}$ method with U6 as the internal control, as performed previously (Yi et al., 2016a).

Schwann cell culture and transfection

Neonatal Sprague-Dawley rats were used to isolate primary Schwann cells as described in detail previously (Li et al., 2015). Isolated Schwann cells were cultured in a complete medium containing Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum (Invitrogen), 1% penicillin and streptomycin (Invitrogen), 2 μM forskolin (Sigma, St. Louis, MO, USA), and 10 ng/mL heregulin (Sigma). Cultured cells were transfected with miR-3099 mimic (miR-3099), non-targeting mimic control, miR-3099 inhibitor (Anti-miR-3099), or non-targeting inhibitor control (RiboBio, Guangzhou, China) using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions.

Cell proliferation assay

Schwann cells were resuspended in a complete medium and seeded at a density of 2×10^5 cells/mL onto 96-well plates pre-coated with poly-L-lysine. Cell proliferation was determined by using Cell-Light EdU DNA Cell Proliferation Kit (RiboBio).

Schwann cells were incubated for an additional 24 hours after the application of 50 μM EdU and fixed with 4% paraformaldehyde. Images were taken using DMR fluorescence microscope (Leica Microsystems, Bensheim, Germany). Cell proliferation rates were determined by counting the numbers of EdU-positive cells and all cells using Image-Pro Plus (Media Cybernetics, Inc., Rockville, MD, USA).

Cell migration assay

Schwann cells were resuspended in DMEM only medium and seeded at a density of 3×10^5 cells/mL onto the top chamber of 6.5 mm transwell chamber with 8-μm pores (Costar, Cambridge, MA, USA). Complete medium containing 10% fetal bovine serum was added to the bottom chamber of the transwell. Schwann cells were incubated for additional 24 hours. The upper surface of the top chamber of transwell was cleaned with a cotton swab and the bottom surface of the top chamber was stained with crystal violet. Images were taken using DMR inverted microscope (Leica). Crystal violet labeled migrated Schwann cells were washed out by 33% acetic acid and the absorbance of crystal violet was measured at 570 nm (Bio-Tek). Cell migration abilities were determined by the absorbance of crystal violet and normalized to the non-targeting control.

Bioinformatic assay

Potential target genes of miR-3099 were analyzed using miRNA target prediction software TargetScan (http://www.targetscan.org/vert_71/). Genes that were functionally related to cell proliferation or migration were selected using Ingenuity pathway analysis software (Ingenuity Systems Inc., Redwood City, CA, USA). Genes that showed a rough negative expression pattern with miR-3099 were screened based on a previously performed microarray analysis (Li et al., 2013). The intersections of TargetScan and Ingenuity pathway analysis-identified genes and negatively expressed genes were obtained using the Venny 2.1.0 online software (http://bioinfogp.cnb.csic.es/tools/venny/index.html). These were considered the potential target genes of miR-3099.

Statistical analysis

Data are presented as the mean ± SEM from three replications. Statistical analysis and graphs were made with GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA, USA). Student's *t*-tests and one-way analysis of variance followed by Dunnett's *post hoc* test were conducted as appropriate. $P < 0.05$ was considered as statistically significant.

Results

Up-regulated miR-3099 in the sciatic nerve segments following sciatic nerve injury

Real-time RT-PCR was performed to validate outcomes from previously performed Solexa sequencing (Li et al., 2011). Real-time RT-PCR data showed that compared with the day 0 control, miR-3099 expression had significantly increased at 1 day following sciatic nerve injury and remained elevated at 4, 7, and 14 days (**Figure 1**). This was consistent with the Solexa sequencing data and demonstrated that miR-3099 was up-regulated following rat sciatic nerve injury.

miR-3099 promotes Schwann cell proliferation

The biological function of miR-3099 was then determined by transfecting Schwann cells with the mimic or the inhibitor of miR-3099. Transfection of Schwann cells with miR-3099 mimic induced a robustly higher proliferation rate compared with transfection with the mimic control (**Figure 2A**). This indicated that an elevated abundance of miR-3099 played a promoting effect on Schwann cell proliferation. On the contrary, transfection of Schwann cells with a miR-3099 inhibitor significantly reduced the proliferation rate when compared with transfection with inhibitor control (**Figure 2B**). This demonstrated that a reduced amount of miR-3099 had an inhibitory effect on Schwann cell proliferation.

Identification of proliferation-related potential target genes of miR-3099

The potential target genes of miR-3099 were investigated using TargetScan software to screen genes containing reserve complementary sequences with the sequence of miR-3099 (5′–TAG GCT AGA AAG AGG TTG GGG A-3′). TargetScan searching obtained a total of 1911 potential candidate genes. Subsequently, a total of 7712 genes related to cell proliferation were selected using the genetic function analysis of Ingenuity pathway analysis software. A total of 605 candidate target genes were identified in the overlapping domains of the Venn diagram. The expression patterns of these 605 genes were further studied using previously obtained microarray data (Li et al., 2013). Genes that were down-regulated were further selected since their expression patterns were negatively related to that of miR-3099. A total number of 11 genes were identified as proliferation-related potential target genes of miR-3099 (**Figure 3A**). The expression patterns of these potential candidate genes, including Nlgn1, Tnmd, Zbtb16, Ppp2r2b, Lsamp, Klf9, Trpc4, Slc25a27, Aqp4, Tnfsf15, and St8sia2, were shown in a heatmap (**Figure 3B**) and the descriptions of their genes were listed in **Figure 3C**.

miR-3099 promotes Schwann cell migration

The role of miR-3099 on Schwann cell migration was also investigated. Schwann cells transfected with the mimic or the inhibitor of miR-3099 were seeded onto transwell chambers to examine whether cell transfection would affect the migration ability of Schwann cells. Compared with the mimic control transfected cells, Schwann cells transfected with miR-3099 exhibited enhanced migration ability (**Figure 4A**). Conversely, compared with cells transfected with mimic control, Schwann cells transfected with miR-3099 inhibitor exhibited suppressed migration ability (**Figure 4B**). These outcomes suggested that miR-3099 promoted the migration of Schwann cells.

Identification of migration-related potential target genes of miR-3099

We also investigated the potential target genes of miR-3099 that were related with cell migration. Ingenuity pathway analysis bioinformatic study suggested that a total of 4202 genes had a cell migration function. Among these genes, 320 genes were predicted by TargetScan as potential target genes. Genes exhibiting down-regulated expression levels were further selected based on microarray outcomes (Li et al., 2013) and overlapping genes in these three sets were collected. A total

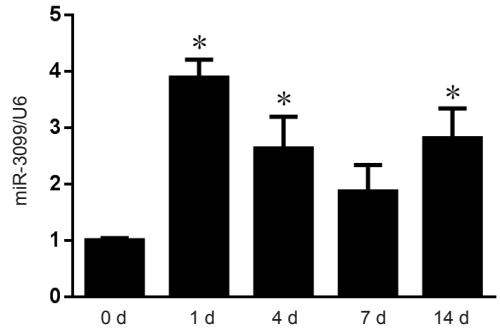


Figure 1 Expression patterns of miR-3099 following sciatic nerve injury.
The expression levels of miR-3099 in the proximal sciatic nerve segments were elevated at 1, 4, 7, and 14 d following sciatic nerve injury. ∗$P < 0.05$, *vs.* 0 d (mean ± SEM, n = triplicate wells from three independent assays; one-way analysis of variance followed by Dunnett's *post hoc* test). d: Day(s).

number of six genes, Astn1, Plc11, Aqp4, St8sia2, Tnfsf15, and Zbtb16, were identified as migration-related potential target genes of miR-3099 (**Figure 5A**). The expression levels (**Figure 5B**) and descriptions are listed in **Figure 5C**.

Discussion

In the current study, miR-3099 expression in the sciatic nerve stumps of rat sciatic nerve injury model was determined at 0, 1, 4, 7, and 14 days after nerve injury. Our results found that miR-3099 was markedly up-regulated after nerve injury. The sciatic nerve stumps contain many types of cells, including Schwann cells, fibroblasts, and macrophages (Gaudet et al., 2011; Jessen et al., 2015; Wang et al., 2017). Of these, Schwann cells are in the majority (Chen et al., 2005; Boerboom et al., 2017) and play critical biological

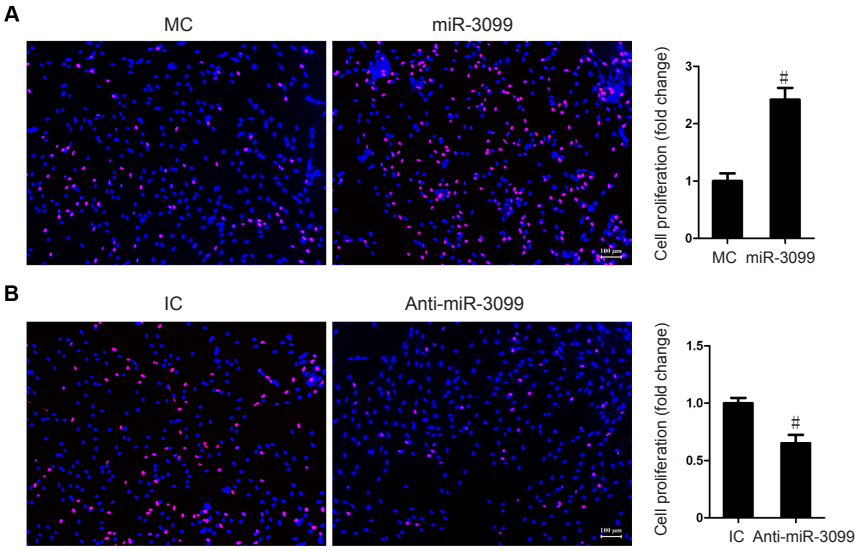
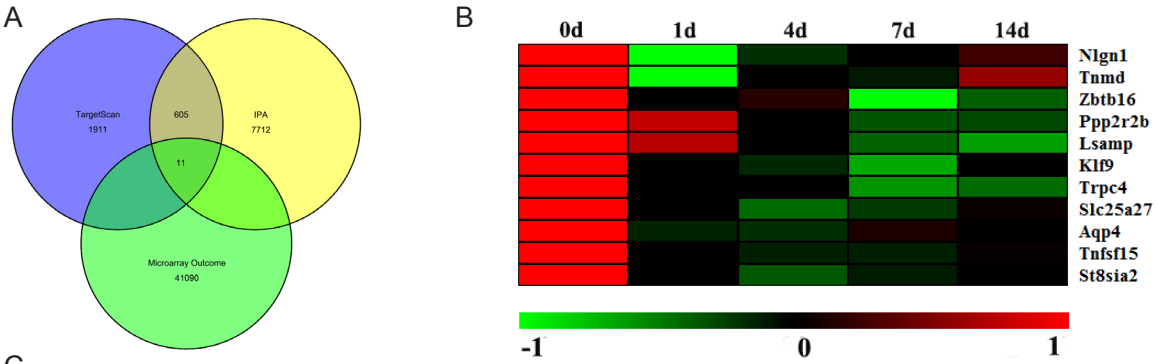


Figure 2 miR-3099 promotes Schwann cell proliferation.
(A) Schwann cells transfected with miR-3099 mimic (miR-3099) exhibited higher proliferation rate of Schwann cells than cells transfected with MC). (B) Schwann cells transfected with miR-3099 inhibitor (Anti-miR-3099) exhibited lower proliferation rate of Schwann cells than cells transfected with IC. Blue shows Hoechst 33342 staining of cell nuclei and red represents EdU-positive cells. Scale bars: 100 μm. #$P < 0.05$, *vs.* MC or IC (mean ± SEM, n = triplicate wells from three independent assays; Student's t-test). MC: Mimic control; IC: inhibitor control.



C

Proliferation-related potential targets of miR-3099	
Targets	Description
Nlgn1	neuroligin 1
Tnmd	tenomodulin
Zbtb16	zinc finger and BTB domain containing 16
Ppp2r2b	protein phosphatase 2, regulatory subunit B, beta
Lsamp	limbic system-associated membrane protein
Klf9	Kruppel-like factor 9
Trpc4	transient receptor potential cation channel, subfamily C, member 4
Slc25a27	solute carrier family 25, member 27
Aqp4	aquaporin 4
Tnfsf15	tumor necrosis factor (ligand) superfamily, member 15
St8sia2	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2

Figure 3 Cell proliferation related potential target genes of miR-3099.
(A) Schematic diagram of the analytical procedures of the identification of potential target genes. (B) Heatmap of differentially expressed genes. The expression patterns of potential target genes were indicated by different colors. Red color indicates up-regulated genes and green color indicates down-regulated genes. (C) The list of potential target genes. d: Day(s).

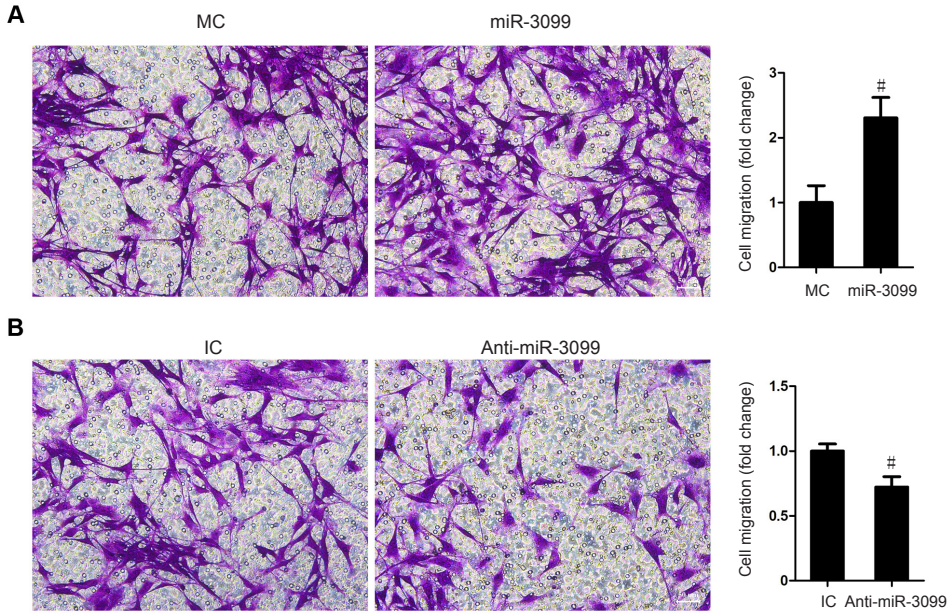
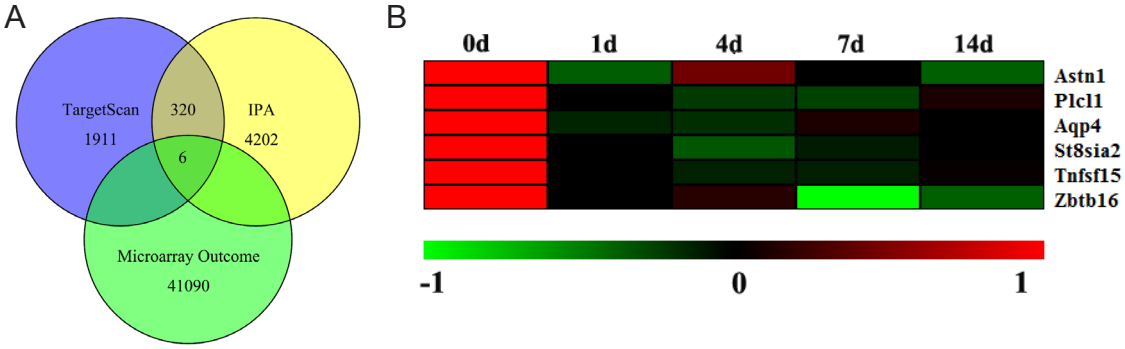


Figure 4 Effect of miR-3099 on Schwann cell migration.
(A) Schwann cells transfected with miR-3099 mimic (miR-3099) exhibit higher migration ability of Schwann cells than cells transfected with mimic control (MC). (B) Schwann cells transfected with miR-3099 inhibitor (anti-miR-3099) exhibit lower migration ability of Schwann cells than cells transfected with inhibitor control (IC). Schwann cells stained with crystal violet. Scale bars: 50 μm. #$P < 0.05$, *vs.* MC or IC (mean ± SEM, n = triplicate wells from three independent assays; Student's t-test).



C

Migration-related potential targets of miR-3099	
Targets	Description
Astn1	astrotactin 1
Plcl1	phospholipase C-like 1
Aqp4	aquaporin 4
St8sia2	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2
Tnfsf15	tumor necrosis factor (ligand) superfamily, member 15
Zbtb16	zinc finger and BTB domain containing 16

Figure 5 Cell migration related potential target genes of miR-3099.
(A) Schematic diagram of the analytical procedures of the identification of potential target genes. (B) Heatmap of differentially expressed genes. The expression patterns of potential target genes were indicated by different colors. Red color indicates up-regulated genes and green color indicates down-regulated genes. (C) The list of potential target genes. d: Day(s).

roles during peripheral nerve regeneration (Bhatheja and Field, 2006; Sullivan et al., 2016; Gonzalez-Perez et al., 2018). After peripheral nerve injury, Schwann cells proliferate and migrate to the injured site, clear away axon and myelin fragments, and build a regenerative path for the elongation of axons (Madduri and Gander, 2010; Glenn and Talbot, 2013; Heinen et al., 2013; Oh et al., 2018). Because of their importance, we determined the biological effects of miR-3099 on Schwann cells by EdU cell proliferation assay and transwell-based cell migration assay. Our results showed that miR-3099 mimic increased Schwann cell proliferation and migration, whereas miR-3099 inhibitor decreased Schwann cell proliferation and migration. The elevated miR-3099 immediately after peripheral nerve injury might promote the proliferation and migration of Schwann cells and thus contribute to the repair and regeneration of injured nerves. In addition to the effect on proliferation and migration, the remyelination of Schwann cells is also essential for peripheral nerve reconstruction. Since miR-3099 remained elevated after peripheral nerve injury, it might also affect Schwann cell remyelination. Further studies could be conducted to examine whether miR-3099 mimic or miR-3099 inhibitor would affect myelin formation. Since other cell types are also present in the sciatic nerve stumps, miR-3099 might also

play a role in them, affecting their biological functions after peripheral nerve injury.

In addition to our functional analysis of miR-3099, we discovered potential target genes of miR-3099 using a combination of bioinformatic tools and high-throughput screen. Nlgn1, Tnmd, Zbtb16, Ppp2r2b, Lsamp, Klf9, Trpc4, Slc25a27, Aqp4, Tnfsf15, and St8sia2 were potential candidate genes that were functionally related to cell proliferation. Astn1, Plc11, Aqp4, St8sia2, Tnfsf15, and Zbtb16 were potential candidate genes that were functionally related to cell migration. Four of these genes, Aqp4, St8sia2, Tnfsf15, and Zbtb16, overlapped for both functions. Considering that miR-3099 affected both the proliferation and migration of Schwann cells, these four overlapping genes might be candidate targets of miR-3099.

Aqp4 encoded a water transport protein aquaporin-4, which conducts water through the cell membrane. Aqp4 is strongly associated with the pathophysiology and pathogenesis of central nerve injury (Oklinski et al., 2016). Besides its critical roles in the central nerve system, Aqp4 expression levels were found to be down-regulated following lesions to the rat optic and sciatic nerves (Zickler et al., 2010). Additionally, another water channel protein of the aquaporin family, aquaporin-1, was demonstrated to be involved in dorsal root ganglion growth and regeneration (Zhang and Verkman, 2015). St8sia2 encoded alpha-2, 8-sialyltransferase 8B, an enzyme that catalyzes the transfer of sialic acid from CMP-sialic acid to N-linked oligosaccharides and/or glycoproteins. St8sia2 was demonstrated to be necessary for the stimulation of axon growth by N-propionylmannosamine (Koulaxouzidis et al., 2015). Tnfsf15 encodes for vascular endothelial growth inhibitor and Zbtb 16 encodes for zinc finger and BTB domain-containing protein 16. There have been no reports on the direct effect of Tnfsf15 or Zbtb 16 on peripheral nerve regeneration. Our studies indicated that Aqp4, St8sia2, Tnfsf15, and Zbtb 16 might be regulated by miR-3099 therefore could be involved in Schwann cell proliferation and migration during peripheral nerve regeneration. Further dual-luciferase assay will be performed to test whether these potential target genes directly bind to miR-3099. siRNA transfection and rescue assays will be performed to determine the biological functions of these genes on Schwann cells.

We have identified a new miRNA in rat Schwann cells, miR-3099, along with some of its biological functions and gene targets. Our study may help to understand the mechanisms behind the biological process of peripheral nerve regeneration.

Author contributions: *Study design and concept: TMQ; experiment implementation: QYL, YM, XHW, PW, ZCC, and TMQ; data analysis: QYL, YM, and TMQ; reagent/material/analysis tool provision and manuscript writing: TMQ. All authors approved the final version of the paper.*
Conflicts of interest: *The authors declare that there are no conflicts of interest associated with this manuscript.*
Financial support: *This study was supported by the Postgraduate Research & Practice Innovation Program of Jiangsu Province of China, No. KYCX17-1910 (to QYL); and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, China (PAPD). The funding sources had no role in study conception and design, data analysis or interpretation, paper writing or deciding to submit this paper for publication.*
Institutional review board statement: *All animal surgery procedures were performed in accordance with Institutional Animal Care guidelines of Nantong University and ethically approved by the Administration Committee of Experimental Animals, Jiangsu, China (license number of The Tab of Animal Experimental Ethical Inspection: 20170302-016). All experimental procedures described here were in accordance with the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals.*

Data sharing statement: *Datasets analyzed during the current study are available from the corresponding author on reasonable request.*
Plagiarism check: *Checked twice by iThenticate.*
Peer review: *Externally peer reviewed.*

Open peer reviewer: *George M. Smith, Temple University, USA.*
Additional file: *Open peer review report 1.*

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P-Reviewer: Smith GM; C-Editor: Zhao M; S-Editors: Wang J, Li CH; L-Editors: Qiu Y, Song LP; T-Editor: Liu XL