

miR-30c promotes Schwann cell remyelination following peripheral nerve injury

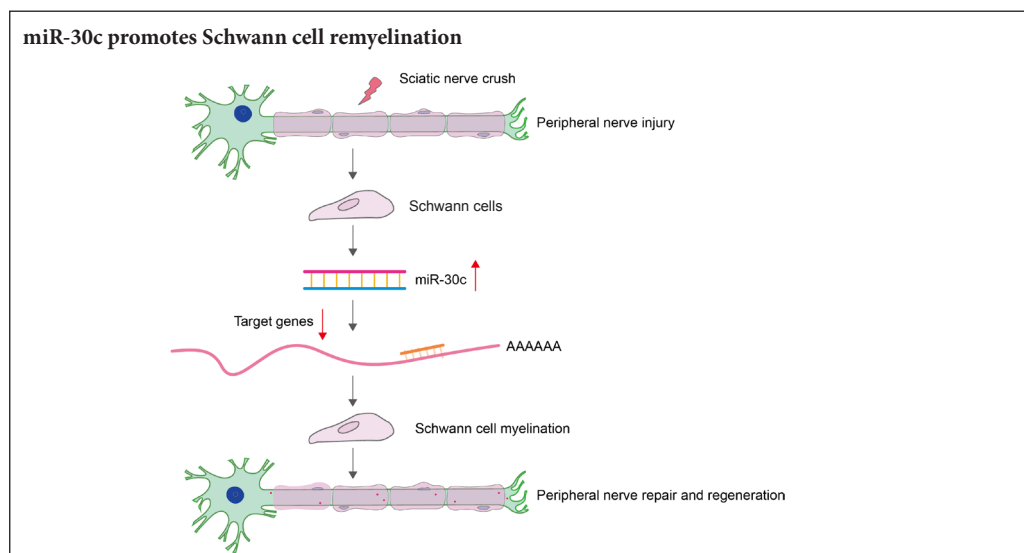
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How to cite this article: Yi S, Wang QH, Zhao LL, Qin J, Wang YX, Yu B, Zhou SL (2017) miR-30c promotes Schwann cell remyelination following peripheral nerve injury. *Neural Regen Res* 12(10):1708-1715.

Funding: This study was supported by the Natural Science Foundation of Jiangsu Province, China, No. BK20150409; the Natural Science Foundation of Jiangsu Higher Education Institutions of China, No. 15KJB180013; the Natural Science Foundation of Nantong of Jiangsu Province, No. MS12015043; Postdoctoral Science Foundation of China, No. 2016M600435; Postdoctoral Science Foundation of Jiangsu Province of China, No. 1601056A; and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Graphical Abstract



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doi: 10.4103/1673-5374.217351

Accepted: 2017-06-02

Abstract

Differential expression of miRNAs occurs in injured proximal nerve stumps and includes miRNAs that are firstly down-regulated and then gradually up-regulated following nerve injury. These miRNAs might be related to a Schwann cell phenotypic switch. miR-30c, as a member of this group, was further investigated in the current study. Sprague-Dawley rats underwent sciatic nerve transection and proximal nerve stumps were collected at 1, 4, 7, 14, 21, and 28 days post injury for analysis. Following sciatic nerve injury, miR-30c was down-regulated, reaching a minimum on day 4, and was then upregulated to normal levels. Schwann cells were isolated from neonatal rat sciatic nerve stumps, then transfected with miR-30c agomir and co-cultured *in vitro* with dorsal root ganglia. The enhanced expression of miR-30c robustly increased the amount of myelin-associated protein in the co-cultured dorsal root ganglia and Schwann cells. We then modeled sciatic nerve crush injury *in vivo* in Sprague-Dawley rats and tested the effect of perineural injection of miR-30c agomir on myelin sheath regeneration. Fourteen days after surgery, sciatic nerve stumps were harvested and subjected to immunohistochemistry, western blot analysis, and transmission electron microscopy. The direct injection of miR-30c stimulated the formation of myelin sheath, thus contributing to peripheral nerve regeneration. Overall, our findings indicate that miR-30c can promote Schwann cell myelination following peripheral nerve injury. The functional study of miR-30c will benefit the discovery of new therapeutic targets and the development of new treatment strategies for peripheral nerve regeneration.

Key Words: nerve regeneration; peripheral nerve regeneration; peripheral nerve injury; sciatic nerve; miRNAs; miR-30c; dedifferentiation; Schwann cells; myelination; *in vivo*; *in vitro*; neural regeneration

Introduction

Injured peripheral nerves retain certain regenerative abilities (Gu et al., 2010; Liu et al., 2011), which is in contrast to the very limited capacity of central nervous system neurons to regenerate. However, full recovery of peripheral nerves after severe injury is impossible (Geuna et al., 2013). Successful

regeneration of injured nerves requires the intrinsic regenerative capability of the neurons and a suitable microenvironment (Chen et al., 2007; Barrette et al., 2008).

Schwann cells are the main glial cells in the peripheral nervous system and are involved in the formation of Bungers bands, the secretion of growth factors, cytokines, and neuro-

trophic factors. They also orchestrate the remyelination of axons; therefore, they establish a permissive local microenvironment for nerve regeneration (Chen et al., 2005; Weber et al., 2010; Wang et al., 2013). It is worth noting that the cellular states of Schwann cells are different in the distinct phases following peripheral nerve injury. In the acute phase post injury, Schwann cells dedifferentiate into stem-like cells, proliferate and migrate to guide axonal regrowth (Freidin et al., 2009; Quintes et al., 2016). After the initiation of axonal elongation, dedifferentiated Schwann cells undergo redifferentiation and remyelination, further participating in the regeneration of myelin sheath (Sundem et al., 2016; Tian et al., 2016). The phenotypic changes of Schwann cells are regulated by a variety of coding and non-coding genes (Xie and Eggan, 2016).

MicroRNAs (miRNAs) are short non-coding RNAs that negatively regulate their target genes at the post-transcriptional level by directly binding to the 3'-untranslated region of complementary mRNAs (Ambros, 2004; Bartel, 2015). A large number of miRNAs are differentially expressed following peripheral nerve injury and they play important roles in modulating the phenotype of Schwann cells (Bremer et al., 2010; Yu et al., 2010). Based on previously obtained miRNA microarray data, we identified 77 differentially expressed miRNAs in injured proximal nerve stumps. According to their expression patterns, these miRNAs were clustered into four patterns: patterns 6, 26, 76, and 9 (Yu et al., 2012a). In pattern 9, miRNAs were down-regulated in the acute phase post injury and were then up-regulated at later time points. This temporal expression pattern was similar to the timeline of Schwann cell redifferentiation indicating that dysregulated miRNAs in this pattern might be involved in the redifferentiation of Schwann cells and the myelination process. Involvement of miRNAs in nerve myelination was recently shown by independent studies of Dicer1-deficient Schwann cells (Pereira et al., 2010; Verrier et al., 2010; Yun et al., 2010; Viader et al., 2011).

We focused here on miR-30c, the miRNA with the most significantly changed expression in pattern 9. We studied its involvement in peripheral nerve injury and regeneration, so as to search for a new treatment strategy for peripheral nerve injury.

Materials and Methods

Ethics statement

All the experimental procedures involving animals were ethically approved by the Administration Committee of Experimental Animals, Jiangsu Province, China (No. 20150304-003) and were used in accordance with Institutional Animal Care guidelines of Nantong University.

Animals

All experimental animals were obtained from the Experimental Animal Center of Nantong University (No. SCXK [Su] 2014-0001 and SYXK [Su] 2012-0031) and were housed in large temperature- and humidity-controlled cages with sawdust bedding. Animals had free access to tap water and

food *ad libitum*. We used 54 adult, male Sprague-Dawley rats weighing 180–200 g; 42 rats were used for the sciatic nerve transection model, and 12 were used for the *in vivo* experiment. Schwann cells were obtained from 30 neonatal 1-day-old Sprague-Dawley rats of either sex. Dorsal root ganglia (DRGs) were isolated from embryonic 15 day Sprague-Dawley rats of either sex.

Sciatic nerve transection

Thirty-six Sprague-Dawley rats were subjected to surgical transection of sciatic nerves. Briefly, each rat was anaesthetized by injection of a mixture of 85 mg/kg trichloroacetaldehyde monohydrate (RichJoint, Shanghai, China), 42 mg/kg magnesium sulfate (Xilong Scientific, Guangzhou, China), and 17 mg/kg sodium pentobarbital (Sigma-Aldrich, St. Louis, MO, USA). The sciatic nerve was exposed and lifted through a 30 mm-long incision on the lateral side of the mid-thigh of the left hind limb. A 10 mm-long nerve segment was excised at the site just proximal to the division of tibial and common peroneal nerves. Six rats were sacrificed by decapitation at each time-point of 1, 4, 7, 14, 21, and 28 days post injury, and 5 mm-long proximal nerve stumps were collected. Sham-operated rats (with sciatic nerves exposed but uninjured) served as controls and were designated as 0 days post-sciatic nerve injury.

RNA extraction, microarray and bioinformatic analysis

Total RNA was extracted from proximal nerve stumps using a mirVana™ miRNA isolation kit (Ambion, Austin, TX, USA) or Trizol™ reagent (Life Technologies, Carlsbad, CA, USA) for subsequent miRNA and mRNA microarray analyses. The quality of isolated RNA samples was evaluated using an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, USA) and then quantified using a NanoDrop ND-1000 spectrophotometer (Infinigen Biotechnology, City of Industry, CA, USA). Genome-wide miRNA and mRNA expression patterns were determined and analyzed by microarray as previously described (Zhou, et al., 2014). Potential mRNA targets of miRNAs in pattern 9 were predicted by integrating putative miRNA targets from the miR-Base database (<http://www.mirbase.org/>) with differentially expressed mRNAs identified from the mRNA microarray analysis. These mRNA targets were further analyzed using Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatic resources (<https://david.ncicrf.gov/>) to enrich for significant Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

Quantitative real-time PCR

RNA isolated from proximal nerve stumps was reverse transcribed using a TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and stem-loop RT primers (Ribobio) to detect the expression levels of miR-30c. For quantification of gene expression, real-time PCR was conducted using SYBR Premix Ex Taq (Tli RNaseH Plus) (TaKaRa) and a StepOnePlus real-time PCR system (Applied Biosystems). The relative expression levels

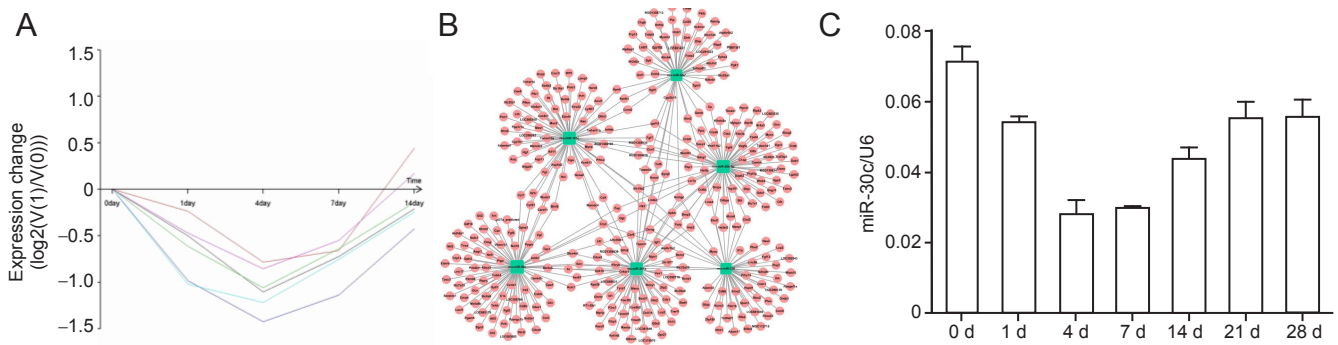


Figure 1 Changes in miRNA expression in proximal nerve stumps following sciatic nerve transection

(A) The expression patterns of six miRNAs in pattern 9 were obtained after bioinformatic analysis at 0, 1, 4, 7, and 14 days post sciatic nerve transection. Red: miR-181c; purplish red: miR-20b-5p; light green: miR-30e; dark green: miR-30c; sky blue: miR-301a; dark blue: miR-338. (B) The regulatory network among miRNAs in pattern 9 and their 347 target genes. Green and red nodes indicate miRNA and mRNA, respectively. (C) Quantitative real-time PCR of miR-30c expression showing initial downregulation in proximal nerve stumps following sciatic nerve transection and then gradual upregulation to near normal levels. miRNA: MicroRNA; d: day(s).

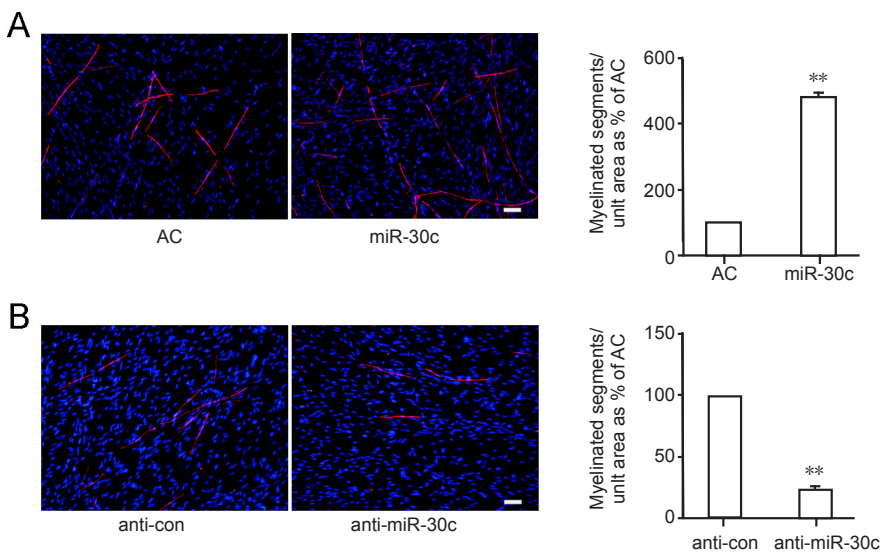


Figure 2 Immunostaining of miR-30c-mediated myelination of Schwann cells

(A) Transfection with miR-30c agomir elevated myelin basic protein expression in co-cultured dorsal root ganglia and Schwann cells. Quantified results of myelinated segments in co-cultured cells transfected with miR-30c agomir (miR-30c) or agomir control (AC) are shown in the histogram. (B) Transfection with miR-30c inhibitor decreased myelin basic protein expression in co-cultured dorsal root ganglia and Schwann cells. Quantified results of the myelinated segments in co-cultured cells transfected with miR-30c inhibitor (anti-miR-30c) or inhibitor control (anti-con) are shown in the histogram. Blue shows Hoechst 33342 staining of cell nuclei and red represents myelin basic protein immunostaining. Bars: 50 μ m. *n* = triplicate wells from three independent assays. ** *P* < 0.01 (Student's *t*-test).

were quantified by the $2^{-\Delta\Delta Ct}$ method.

In vitro experiment

Sciatic nerve sections were collected from 30 neonatal 1-day-old Sprague-Dawley rats after anaesthetized by injection of a mixture of 85 mg/kg trichloroacetaldehyde monohydrate (RichJoint), 42 mg/kg magnesium sulfate (Xilong Scientific), and 17 mg/kg sodium pentobarbital (Sigma-Aldrich). The skin was incised on the lateral side of the hind limb, the sciatic nerve exposed, and an 8 mm-long section of the nerve collected. Primary Schwann cells were isolated from the nerve sections by using trypsin and then treated with anti-Thy1.1 antibody (Sigma) and rabbit complement (Invitrogen, Carlsbad, CA, USA) to remove fibroblasts (Mantuano et al., 2008). This procedure has previously been shown to produce a cell population of > 98% Schwann cells, as indicated by staining with the Schwann cell marker, anti-S100 (DAKO, Carpinte-

Table 1 Enriched KEGG pathways of intersected genes

KEGG ID	Pathway	Count	<i>P</i> value
4610	Complement and coagulation cascades	9	5.77E-05
4060	Cytokine-cytokine receptor interaction	16	6.73E-05
5150	Staphylococcus aureus infection	6	0.001
4080	Neuroactive ligand-receptor interaction	16	0.002
4020	Calcium signaling pathway	11	0.008
4640	Hematopoietic cell lineage	6	0.015
52	Galactose metabolism	3	0.022
4672	Intestinal immune network for IgA production	4	0.024
2010	ABC transporters	4	0.027
4966	Collecting duct acid secretion	3	0.031

KEGG ID, pathway name, the number of differentially expressed genes annotated in the specific KEGG pathway, and the *P* value for each KEGG pathway with a *P* value < 0.05.

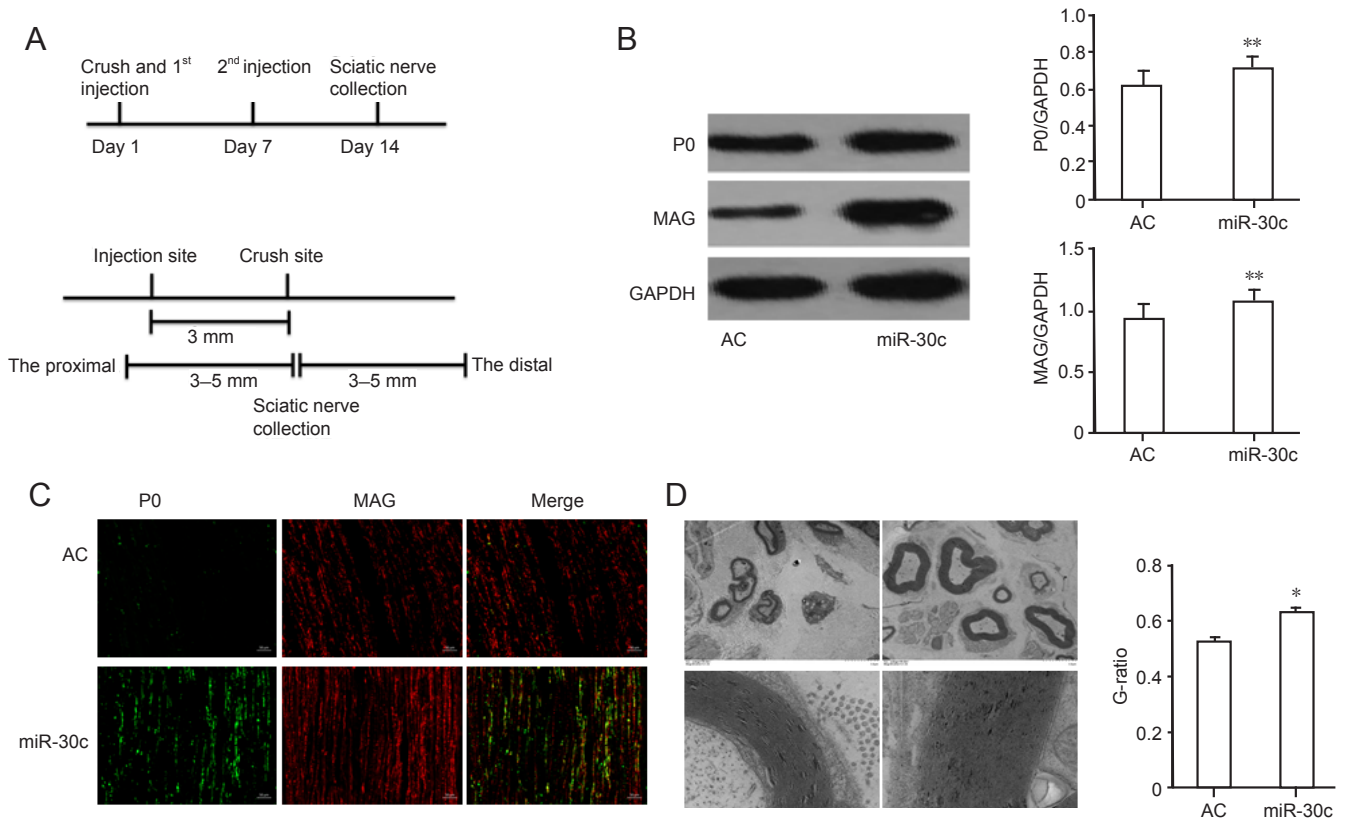


Figure 3 miR-30c promoted myelination of injured sciatic nerve *in vivo*.

(A) Schematic depiction of the *in vivo* miR-30c study. (B) Western blot analysis of P0 and MAG protein levels (myelin-associated proteins) in the sciatic nerve of rats injected with miR-30c agomir (miR-30c) or negative control (AC). (C) Immunohistochemistry of P0 and MAG proteins. Green indicates P0 and red indicates MBP. Bars: 50 μ m. (D) Representative transmission electron microscopy photomicrographs and analysis of myelinated nerve fibers. The lower two figures are higher magnifications of the upper two figures. Bars: 5.0 μ m in the upper two figures, bars: 500 nm in the lower two figures. * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test). MAG: Myelin-associated glycoprotein; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

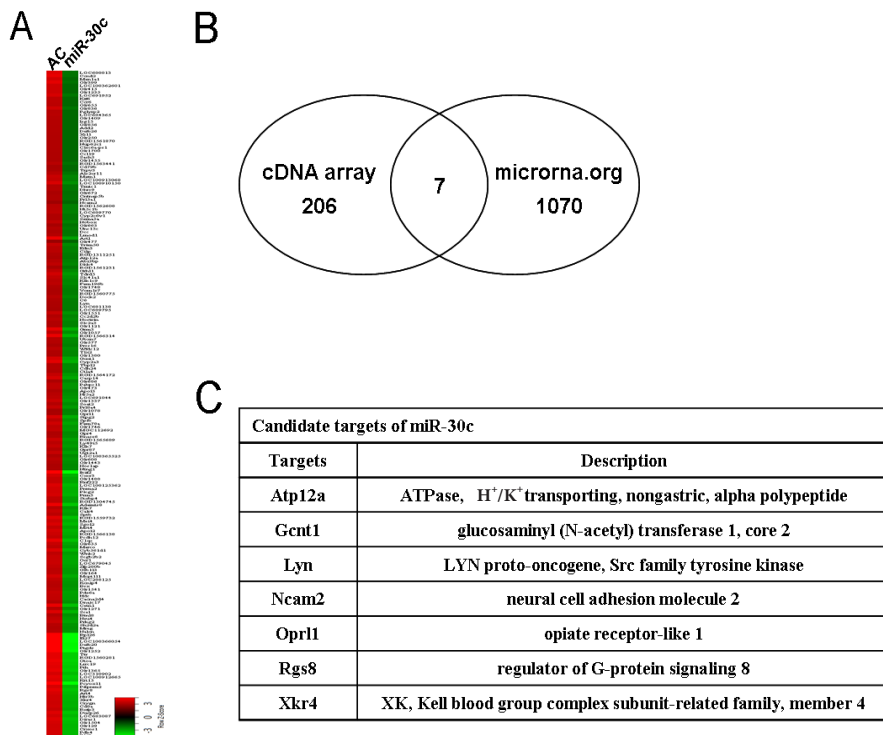


Figure 4 Identification of candidate miR-30c targets.

(A) Heatmap of down-regulated mRNAs in response to miR-30c over-expression. The expression levels of mRNAs were determined by cDNA sequencing and mRNAs that were down-regulated more than 2-fold in Schwann cells transfected with miR-30c are listed. (B) The intersection of down-regulated mRNAs and predicted target genes of miR-30c. (C) The list of candidate miR-30c targets.

ria, CA, USA) (Yi et al., 2016a). Primary Schwann cells were passaged no more than three times prior to use and were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Invitrogen) in a humidified 5% CO₂ incubator at 37°C.

Purified Schwann cells were transfected with miR-30c agomir (20 nmol/L; Ribobio, Guangzhou, Guangdong Province, China), negative control (20 nmol/L; Ribobio), miR-30c inhibitor (100 nmol/L; Ribobio), or inhibitor control (100 nmol/L; Ribobio) over 3 days using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions (Zhou, et al., 2014). Agomir is very stable and is suitable for long-term miRNA gain of function studies both *in vitro* and *in vivo* (Li et al., 2015).

The co-culture of rat DRGs and Schwann cells was performed as previously described (Saitoh and Araki, 2010). Briefly, DRGs were isolated from embryonic day 15 Sprague-Dawley rats, digested in 1% collagenase, and plated on 24-well plates coated with extracellular matrix laminin (Invitrogen) and poly-L-lysine (Sigma) at 2×10^5 cells per well in neurobasal medium supplemented with 2% B27 and 100 ng/mL nerve growth factor. Transfected Schwann cells (3×10^5 cells per well) were then plated onto established neuronal cultures at a DRG: Schwann cell ratio of 1:5 in DMEM supplemented with 10% FBS and 100 ng/mL nerve growth factor. Seven days later, the mixed cultures of Schwann cells and neurons were treated with 50 µg/mL ascorbic acid (Sigma) to induce myelination. Myelination was allowed to proceed for up to 28 days, and the culture medium was renewed every 2–3 days.

In addition, Schwann cells transfected with miR-30c agomir or non-targeting negative control were isolated for cDNA array analysis. mRNAs that were down-regulated more than 2-fold were selected for miR-30c candidate target gene prediction.

***In vivo* experiment**

Twelve adult male Sprague-Dawley rats were anaesthetized and their sciatic nerves were crushed with forceps as previously described (Yi et al., 2015). Rats were randomly divided into two groups ($n = 6$ per group) to receive injection of miR-30c agomir or agomir control. Five nmol of miR-30c agomir or negative control were dissolved in 16 µL saline and injected perineurally at the injury site on the same day and 7 days after nerve crush. Fourteen days after nerve injury, the rats were sacrificed by decapitation and their sciatic nerve stumps were harvested and subjected to immunohistochemistry, western blot analysis, and transmission electron microscopy observation. The miR-30c agomir was directed at the mature miRNA sequence, synthesized by chemical methods, and treated with specific chemical modification methods by Ribobio. It can be applied by systemic or local injection, inhalation delivery, or oral administration and is able to cross cell membranes (Su et al., 2015).

Immunohistochemistry and immunofluorescence

Co-cultured DRGs and Schwann cells and crushed proximal nerve stumps were collected and processed as previously described (Zanazzi et al., 2001). For immunostaining, cells and tissue slices were fixed with 4% paraformaldehyde/PBS for 5 minutes at room temperature, blocked with 5% goat serum for 30 minutes at room temperature and incubated with primary antibodies: anti-myelin basic protein (MBP) (1:500; AB62631, Abcam St Louis, MO, USA), anti-peripheral myelin protein zero (P0) (1:125; AB31851, Abcam), or anti-myelin-associated glycoprotein (MAG) (1:200; AB89780, Abcam) overnight at 4°C. Specimens were then incubated with Cy3-conjugated goat anti-rabbit secondary antibody (1:1,000; Sigma) for 2 hours at room temperature, and were then mounted on microscope slides. Samples were visualized and images captured using an optical and epifluorescence microscope (Axio Imager M2, Carl Zeiss Microscopy GmbH, Jena, Germany). All assays were performed in triplicate.

Western blot analysis

Protein samples were extracted from cultured cells or crushed proximal sciatic nerve stumps, and lysed in Laemmli sample buffer containing 2% SDS, Tris-HCl (52.5 mmol), and protein inhibitors. Protein concentration was determined using a micro BCA protein assay kit (Pierce, Rockford, IL, USA). Extracted protein lysates were mixed with β-mercaptoethanol, glycerin, and bromophenol-blue and then incubated at 95°C for 5 minutes. Equal amounts of protein were then separated by 10% SDS-PAGE and electro-transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked in 5% nonfat dried milk for 2 hours at room temperature, probed with the following primary antibodies: anti-P0 (1:400), anti-MAG (1:600), or anti-GAPDH (1:4,000; AB8245, Abcam) overnight at 4°C, incubated with goat anti-rabbit HRP-conjugated secondary antibodies (1:5,000; Pierce) for 1 hour at room temperature, and then developed with enhanced chemiluminescence reagent (Pierce). Quantification was determined using Quality One software (Bio-Rad, Hercules, CA, USA). All assays were performed in triplicate.

Transmission electron microscopy

The crushed sciatic nerve stumps were harvested, fixed in 4% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated stepwise in increasing concentrations of ethanol, and then embedded in Epon 812 epoxy resin (Sigma). After staining with lead citrate and uranyl acetate, ultrathin sections were observed under a transmission electron microscope (JEOL Ltd., Tokyo, Japan). Images were taken at a magnification of $\times 1.2$ k and $\times 20.0$ k. The diameters of myelinated nerve fibers and corresponding axons were measured using Leica QWin V3 (Leica, Wetzlar, Germany) and the G ratio (axonal diameter/outer diameter of the whole nerve fiber) was calculated.

Table 2 Dysregulated miRNAs in pattern 9 after sciatic nerve transection

Unique ID	P value	False discovery rate	Average of intensities				
			0 day	1 day	4 days	7 days	14 days
rno-miR-30c	1.73E-05	5.19E-05	2,327.2	1,646.6	1,082.8	1,411.5	2,012.7
rno-miR-181c	0.00002	5.82E-05	105.5	89.5	61.4	66.8	143.4
rno-miR-30e	4.11E-05	9.69E-05	148.1	96.8	71.1	95.2	134.3
rno-miR-338	5.84E-05	0.0001	1,3479.9	6,829.3	5,028.9	6,134.5	10,050.3
rno-miR-301a	0.0001	0.0002	359.9	177.4	155.1	213.9	303.7
rno-miR-20b-5p	0.0003	0.0005	77.6	56.1	42.9	53	87.3

Statistical analysis

Statistical analyses were performed using SPSS 21.0 (SPSS, IBM, NY, USA). Student's *t*-test was used for comparison between two groups. A *P* value < 0.05 was considered statistically significant. All data are expressed as the mean ± SEM.

Results

Numerous miRNAs were down-regulated and then up-regulated in nerve stumps after sciatic nerve transection

Pattern 9 contained six dysregulated miRNAs: miR-30c, miR-181c, miR-30e, miR-338, miR-301a, and miR-20b-5p. The expression levels of miRNAs in this pattern were decreased from day 1 post-injury and reached a minimum on day 4. Levels then increased and reached normal levels on day 14 (Figure 1A). We compared miRs in the miRBase database with differentially expressed mRNAs (identified by the adjusted F-test with the Random Variation Model) and identified 347 putative target genes. A regulatory network among miRNAs in pattern 9 and their target genes were constructed, which showed that several mRNA targets were regulated by multiple miRNAs (Figure 1B). KEGG pathway analysis identified critical signaling pathways and biological functions involved in these target genes (Table 1). Among enriched KEGG pathways, cytokine-cytokine receptor interaction, neuroactive ligand-receptor interaction, and calcium signaling pathways include numerous target genes, suggesting that these miRNAs and target mRNAs may regulate neuroprotection and regeneration.

miR-30c was differentially expressed in sciatic nerve stumps at different time points following nerve transection

The microarray intensity readings of these six dysregulated miRNAs were further studied (Table 2). miR-30c and miR-338 exhibited relatively higher intensities, while those of miR-181c, miR-30e, and miR-301a were relatively lower. Statistical analysis suggested that miR-30c was the most significantly changed following sciatic nerve transection. Therefore, miR-30c was further studied.

Firstly, the expression profile of miR-30c was determined by quantitative real-time PCR (Figure 1C). Consistent with the microarray results, the expression level of miR-30c was immediately downregulated post nerve transection, reaching a minimum on day 4, and expression then gradually in-

creased up to 28 days post nerve transection, indicating that miR-30c was downregulated in the acute phase post injury and then slowly returned to normal levels during the regeneration phase.

miR-30c regulated Schwann cell myelination *in vitro*

miR-30c agomir was transfected into cultured Schwann cells to examine its effect on Schwann cell myelination. After the co-culture of Schwann cells with DRGs, myelin sheath began to form. The miR-30c agomir-transfected Schwann cells, showed a higher expression level of MBP (a myelin-associated protein) compared with cells transfected with negative control (Figure 2A). In contrast, cells transfected with miR-30c inhibitor had a reduced expression level of MBP (Figure 2B). These findings indicated that miR-30c exerted a positive effect on Schwann cell myelination.

miR-30c accelerated sciatic nerve myelination *in vivo*

The *in vivo* effect of miR-30c was examined by directly injecting miR-30c agomir into the injury site at 1 and 7 days after sciatic nerve crush (Figure 3A). Western blotting showed that post miR-30c agomir injection, the relative abundance of P0 in sciatic nerve stumps at 14 days after nerve injury appeared to be slightly increased (Figure 3B), together with an overt increase in the relative abundance of myelin-associated glycoprotein (MAG) (Figure 3B). Immunohistochemistry showed that in the control group, the levels of P0 and MAG in the injured sciatic nerves were decreased at 14 days post injury. However, miR-30c agomir robustly increased levels of P0 and MAG (Figure 3C). Morphological changes were identified by transmission electron microscopy. Compared with negative control-injected rats, miR-30c agomir-injected rats exhibited significantly thicker myelin sheaths. The average thicknesses of myelin sheaths in rats injected with agomir control and miR-30c agomir were 2.32 μm (the average outer diameter was 6.31 μm and the average axonal diameter was 3.99 μm), and 3.40 μm (the average outer diameter was 7.06 μm and the average axonal diameter was 3.67 μm), respectively. Furthermore, rats injected with miR-30c agomir revealed a higher G-ratio, indicating that miR-30c promoted the myelination process in the injured sciatic nerve *in vivo* (Figure 3D).

Identification of candidate miR-30c targets

miRNAs function by binding to and regulating their target

genes. We attempted to identify candidate miR-30c target genes by performing cDNA microarray analysis together with MiRanda prediction. Schwann cells transfected with miR-30c agomir yielded 206 genes that were down-regulated more than 2-fold (**Figure 4A**). The miRNA target prediction program, MiRanda, identified 1,070 putative target genes. The joint use of cDNA microarray and MiRanda-based analysis identified seven candidate target genes, *Atp12a*, *Gcnt1*, *Lyn*, *Ncam2*, *Oprl1*, *Rgs8*, and *Xkr4* (**Figure 4B, C**).

Discussion

Successful repair of peripheral nerve injury relies on a series of complicated cellular events, including timely dedifferentiation and redifferentiation of Schwann cells. We previously identified a group of differentially expressed miRNAs (in patterns 6, 26, 76, and 9) in proximal nerve stumps after sciatic nerve injury. Two of the upregulated miRNAs in pattern 76, miR-221/222, advanced Schwann cell proliferation and migration by targeting longevity assurance homologue 2 (*Lass2*) post injury (Yu et al., 2012b). miR-9, a pattern 6 miRNA that was downregulated post injury, inhibited Schwann cell migration by targeting collagen triple helix repeat containing protein 1 (*Cthrc1*) (Zhou et al., 2014). Other studies also indicated regulatory roles of miRNAs in Schwann cells, such as the beneficial effect of miR-340 on Schwann cell migration, miR-1 and miR-sc3 effects on Schwann cell proliferation and migration, and the inhibitory effect of miR-sc8 and Let-7 on Schwann cell proliferation and migration (Gu et al., 2015; Li, et al, 2015, 2017; Yi et al., 2016b).

These findings mainly identified effects of miRNAs on Schwann cell dedifferentiation (proliferation and/or migration), which is critical in the acute phase post peripheral nerve injury. The redifferentiation of Schwann cells is equally important for peripheral nerve regeneration as proper and timely myelin formation and axon wrapping is necessary for functional recovery. Notably, several studies indicate that the differentiation and myelination processes of oligodendrocytes (glial cells in the central nervous system) can be regulated by miRNAs (Dugas et al., 2010; Emery 2010; Zhao et al., 2010). However, the regulatory effect of miRNAs on the differentiation of and myelination by glial cells in the peripheral nervous system remains unknown.

Considering the importance of Schwann cell redifferentiation, miRNAs in pattern 9 attracted our attention. miRNAs in this pattern exhibited a U-shaped expression pattern and might be related to the phenotype switch of Schwann cells. Post nerve injury, long-term up-regulated miRNAs in this pattern might contribute to Schwann cell redifferentiation and myelin sheath formation (Xu et al., 2014; Yu et al., 2015). Here, we studied the dynamic changes and cellular functions of miR-30c in detail. An *in vitro* study showed that miR-30c robustly increased the amount of MBP in co-cultured DRGs and Schwann cells. An *in vitro* study further indicated that miR-30c promoted Schwann cells myelination and thus contributes to nerve regeneration.

Further studies have identified the importance of miR-30c in the nervous system. Sun *et al.* (2016 a, b) showed that

miR-30c promoted the cell cycle of stem cells and enhanced adult neurogenesis in the brain by targeting semaphorin 3A. Li *et al.* (2015) demonstrated that miR-30c protected the spinal cord from ischemia/reperfusion injury. Our study indicated that miR-30c might also play significant roles in the peripheral nervous system. In the future, we will perform luciferase assays, candidate gene transfection, and rescue assays, to determine the direct target gene(s) of miR-30c.

In summary, we show that miR-30c is downregulated following sciatic nerve injury, reaching a minimum on day 4. Gradual recovery of miR-30c expression promotes the myelination of Schwann cells. The functional study of miR-30c provides a better understanding of the regulatory role of miRNAs in peripheral nerve regeneration and might provide new avenues for the treatment of peripheral nerve injury.

Author contributions: SY, BY, SLZ conceived and designed the experiments. SY, QHW, LLZ, JQ, YXW, and SLZ performed the experiments. SY, YXW, BY, and SLZ analyzed the data. SY, BY, and SLZ contributed to reagents/materials/analysis tools. SY, BY, and SLZ wrote the manuscript. All authors have read and approved the final manuscript.

Conflicts of interest: None declared.

Research ethics: The study protocol was approved by Administration Committee of Experimental Animals of Nantong University (20150304-003). All animal care and animal surgeries were performed in accordance with the Guide for Care and Use of Laboratory Animals (Public Health Service, 19965, NIH Publication No. 85-23).

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.

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Open peer reviewer: Shan-hui Hsu, National Taiwan University, China.

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