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RESEARCH ARTICLE

miR-9 inhibits Schwann cell migration by targeting *Cthrc1* following sciatic nerve injury

Songlin Zhou[1,*], Rong Gao[1,*], Wen Hu[1], Tianmei Qian[1], Na Wang[1], Guohui Ding[2], Fei Ding[1], Bin Yu[1,‡] and Xiaosong Gu[1,‡]

ABSTRACT

The regulative effects of microRNAs (miRNAs) on responses of Schwann cells to a nerve injury stimulus are not yet clear. In this study, we noted that the expression of eight miRNAs was downregulated at different time points following rat sciatic nerve transection, and found that 368 potential targets of these eight miRNAs were mainly involved in phenotypic modulation of Schwann cells. Of these miRNAs, miR-9 was identified as an important functional regulator of Schwann cell migration that was a crucial regenerative response of Schwann cells to nerve injury. *In vitro*, upregulated expression of miR-9 inhibited Schwann cell migration, whereas silencing of miR-9 promoted Schwann cell migration. Intriguingly, miR-9 exerted this regulative function by directly targeting collagen triple helix repeat containing protein 1 (CTHRC1), which in turn inactivated downstream Rac1 GTPase. Rac1 inhibitor reduced the promotive effects of anti-miR-9 on Schwann cell migration. *In vivo*, high expression of miR-9 reduced Schwann cell migration within a regenerative nerve microenvironment. Collectively, our results confirmed the role of miR-9 in regulating Schwann cell migration after nerve injury, thus offering a new approach to peripheral nerve repair.

KEY WORDS: miR-9, Schwann cell, Migration, CTHRC1, Peripheral nerve regeneration

INTRODUCTION

Owing to traffic and industrial accidents, natural disaster, war damage and deliberate surgery, people are likely to suffer from traumatic injury to their peripheral nerves (Noble et al., 1998). There has been an increasing incidence worldwide of this common clinical problem in recent years, and transection injury to peripheral nerves results in anatomic interruption of the nerve continuity, thus exerting a devastating impact on patients' quality of life (Gu et al., 2011; Rishal and Fainzilber, 2010). Despite an intrinsic regenerative capacity of the peripheral nervous system (PNS), which is different from the characteristics of the adult central nervous system (CNS), the functional outcome of peripheral nerve repair is always unsatisfactory because although the injured peripheral nerves are able to regrow and reconnect to their targets, their original functioning is seriously compromised (Gruart et al., 2003). This has been assumed to be due to a too low a rate of axonal outgrowth from the injured neuron (Ma et al., 2011). Therefore, a better understanding of the molecular mechanisms underlying peripheral nerve injury and regeneration is crucial for improving the functional outcomes of peripheral nerve repair.

Accumulating evidence has indicated that the ability of axons in injured peripheral nerves to grow toward their targets is mainly dependent on the biological behaviors of Schwann cells, the principal glial cells in the PNS. Following peripheral nerve injury, mature differentiated Schwann cells undergo significant phenotypic modulation, in particular shedding their myelin sheaths and dedifferentiating to a progenitor/stem-cell-like state (Freidin et al., 2009). The dedifferentiated Schwann cells can replenish lost or damaged tissue through proliferation and migration, and can produce a favorable environment for axonal outgrowth through clearing myelin debris and forming cellular conduits or corridors (called bands of Buengner) to guide the directional growth of axons (Parrinello et al., 2010; Yao et al., 2013). Furthermore, many previous studies have described that the phenotypic modulation of Schwann cells is governed by activation of a regulatory network, including the transcription factors Sox2, Sox10, Krox20/Egr2, Oct6/SCIP, Brn2, NFATc4 (Ghislain and Charnay 2006; Kao et al., 2009; Parrinello et al., 2010). The detailed mechanisms that account for dedifferentiation, proliferation, migration and redifferentiation of Schwann cells during axonal regeneration, however, remain largely unclear.

microRNAs (miRNAs) are short, ~22 nucleotide-long, non-coding RNA molecules that negatively regulate the expression of a wide variety of genes mainly through direct interaction with the 3′-untranslated regions (3′-UTR) of their target mRNAs (van Wolfswinkel and Ketting, 2010). The number of putative mRNA targets regulated by miRNAs at the post-transcriptional level is estimated to exceed 60% of total human genes (Friedman et al., 2009), suggesting the pivotal roles of miRNAs in a diverse array of physiological and pathological processes. In comparison to the study of miRNAs in many other biological events, the importance of miRNAs in peripheral nerve injury and regeneration has not been fully investigated. Our previous study using microarray and deep sequencing showed that abnormal expression of miRNAs in dorsal root ganglia was involved in nerve regeneration after transection to rat sciatic nerves (Yu et al., 2011; Zhou et al., 2011). The role of miRNAs in nerve myelination has been investigated by two independent studies on Dicer1-deficient Schwann cells (Pereira et al., 2010; Yun et al., 2010). It was further demonstrated that the majority of miRNAs expressed in mature Schwann cells could maintain the differentiated state of cells, but upon peripheral nerve injury, most of these miRNAs are

[1]Jiangsu Key Laboratory of Neuroregeneration, Nantong University, Nantong 226001, China. [2]Key Lab of Systems Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China.

*These authors contributed equally to this work.

‡Authors for correspondence (yubin@ntu.edu.cn; nervegu@ntu.edu.cn)

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downregulated allowing transcriptionally driven dedifferentiation of the Schwann cells (Viader et al., 2011). Likewise, we found that miR-221/222, belonging to a class of upregulated miRNAs after nerve injury, promoted proliferation and migration of Schwann cells by targeting longevity assurance homologue 2 (Yu et al., 2012). All the above findings suggest that miRNAs are integral components of the regulatory network responsible for the phenotypic modulation of mature Schwann cells.

Our previous miRNA array data indicated that the expression of miR-9 was downregulated after nerve injury, and the change of expression of miR-9 was opposite to that of miR-221/222. Moreover, downregulated expression is considered to be the most important expression change of miRNAs after nerve injury (Yu et al., 2012). In this study, therefore, we aimed to determine the possible involvement of miR-9 in peripheral nerve repair and regeneration. The *in vitro* and *in vivo* results showed that miR-9 inhibited Schwann cell migration by directly targeting collagen triple helix repeat containing protein 1 (CTHRC1), which subsequently inactivated downstream Rac1 GTPase.

RESULTS

Downregulation of miR-9 expression in the proximal nerve stump following sciatic nerve injury

We have previously measured the global miRNA expression profile in the proximal nerve stump after sciatic nerve transection in rats. A total of 77 miRNAs were differentially expressed at 0, 1, 4, 7 and 14 days post injury with four significantly different patterns of expression change, referred to as profile 6, 26, 76 and 9 (Yu et al., 2012). In this study, we focused on the eight miRNAs in profile 6, i.e. let-7a, let-7c, miR-26b, miR-352, miR-100, miR-148b-3p, miR-9* and miR-9. The expression of the eight miRNAs in profile 6 rapidly decreased from 1 day post injury, and the expression was significantly lower at 1, 4, 7 and 14 days post injury than at 0 days post injury (supplementary material Fig. S1 and Table S1). This pattern of downregulated expression was considered the most important among all the patterns of expression change.

We searched for the putative targets of the eight miRNAs in profile 6 using the miRBase database, and then integrated putative miRNA targets with differentially expressed mRNAs to yield 368 potential targets. In order to unravel credible biological functions, we conducted Gene ontology (GO) category and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for the intersected genes. The GO terms having the highest enriched score and the most significant *P*-value are listed in Table 1. We noted that the most significant GO functions were cellular localization-related functions, including establishment of localization, secretion by the cell, and regulation of cellular localization. Functional classification obtained using KEGG analysis (Wnt signaling pathway, cytokine–cytokine receptor interaction and ErbB signaling pathway) was significantly enriched by the targets (supplementary material Table S2). In addition, the network used to link the eight miRNAs with 368 target genes has been built (supplementary material Fig. S2).

Functional study of miR-9

Out of the eight miRNAs in profile 6, miR-9 was exclusively selected for functional studies because of its known migration-inhibitory effects in different cell types (Delaloy et al., 2010; Liu et al., 2012; Zhang et al., 2012). Interestingly, the enriched GO terms (Table 1) mainly included cellular-localization-related functions, which seemed to coincide with the previously known effects of miR-9. To confirm the time-dependent differential expression of miR-9, we applied quantitative real-time polymerase chain reaction (qRT-PCR) to determine the expression of miR-9 in proximal nerve stumps harvested at designated time points post injury. The PCR data provided evidence for the change in expression of miR-9, which was in agreement with microarray data (Fig. 1A). *In situ* hybridization at 4 days post injury also showed that miR-9 expression was downregulated in Schwann cells of proximal nerve stumps (Fig. 1B).

To test the influences of the miR-9 dysregulation on cellular functions, Schwann cells were transfected with miR-9 mimic, miR-9 inhibitor and non-targeting negative controls, respectively. Transwell migration assays showed that transfection of Schwann cells with the miR-9 mimic significantly decreased their migration compared with transfection of the mimic control (Fig. 2A), whereas miR-9 silencing by the miR-9 inhibitor markedly increased Schwann cell migration (Fig. 2B). We also conducted an *in vitro* wound healing assay, which showed that migration of Schwann cells was reduced after transfection with the miR-9 mimic (Fig. 2C), and increased after transfection with the miR-9 inhibitor (Fig. 2D).

In addition, the viability of Schwann cells was assessed using the CCK-8 assay. No changes in cytotoxicity were found after Schwann cells were transfected with miR-9 mimic or miR-9 inhibitor (supplementary material Fig. S3A). The proliferation of Schwann cells was also examined by using 5-ethynyl-2′-deoxyuridine (EdU) incorporation, and miR-9 was found to have no significant effects (supplementary material Fig. S3B). From the above assessments, we excluded the possibility that miR-9 regulated Schwann cell survival and proliferation, and suggested that miR-9 dysregulation directly affected Schwann cell migration.

Table 1. Gene ontology analysis

GO ID	GO terms	Count	*P*-value
GO:0048513	Organ development	77	6.37E-11
GO:0007275	Multicellular organismal development	73	1.98E-08
GO:0009611	Response to wounding	33	4.19E-08
GO:0051234	Establishment of localization	89	1.13E-07
GO:0032940	Secretion by cell	27	2.56E-07
GO:2000026	Regulation of multicellular organismal development	36	2.70E-07
GO:0065008	Regulation of biological quality	53	7.41E-07
GO:0061061	Muscle structure development	21	9.93E-07
GO:0032989	Cellular component morphogenesis	27	1.22E-06
GO:0060341	Regulation of cellular localization	22	1.82E-06

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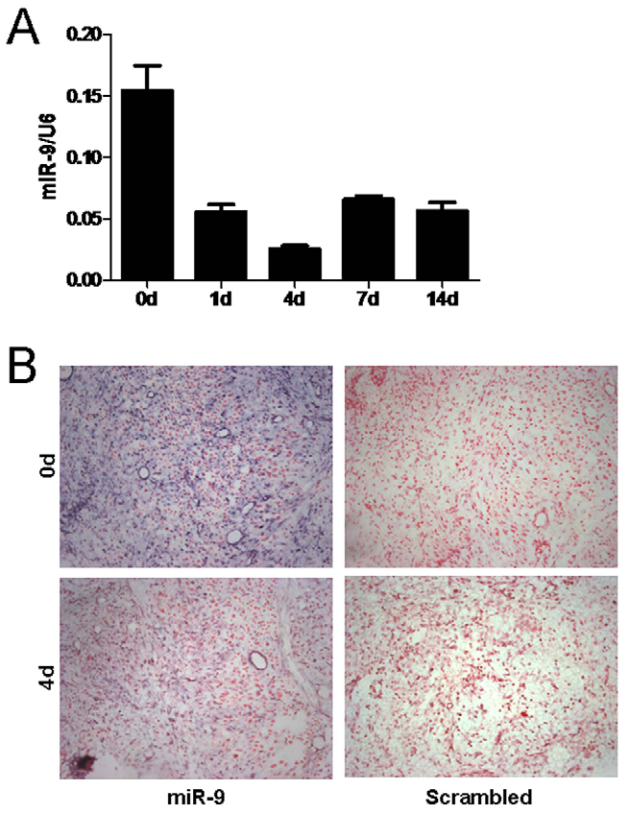

Fig. 1. Downregulation of miR-9 in proximal nerve stumps following sciatic nerve transection. (A) qRT-PCR showed that the miR-9 expression in the proximal nerve stump was decreased at 1, 4, 7 and 14 days post injury compared with control. (B) *In situ* hybridization of proximal nerve stumps performed 4 days post injury showed that miR-9 was downregulated mainly in Schwann cells.

Post-transcriptional downregulation of *Cthrc1* expression by miR-9 targeting its 3′-UTR

To explore how miR-9 inhibits Schwann cell migration, two strategies were used to identify the potential mRNA target(s) of miR-9. (1) We performed microarray analysis to search for genes regulated by miR-9, compared the resulting data with the data from the TargetScan prediction program, and thus identified a total of 35 candidate genes (supplementary material Table S3), among which five genes (*Nuak1*, *Trib3*, *Tjp1*, *Cthrc1* and *Lrrc15*) were found to relate to the promotion of cell migration. (2) We performed correlation analysis between eight miRNAs in profile 6 and 368 targets, and then selected and identified the 14 inversely correlated miRNA–gene target pairs (strong correlation: $\rho<-0.7$; supplementary material Table S4). By combining the results obtained from these two strategies, we noted that the intersection between five cell-migration-related genes and the genes from 14 miRNA–gene target pairs yielded one overlapped gene, namely *Cthrc1*, which might be potentially downregulated by miR-9.

qRT-PCR (Fig. 3A) and western blotting (Fig. 3B) indicated that after nerve injury, an inverse change tendency existed between CTHRC1 expression (at the mRNA or protein level) and miR-9 expression. Briefly, down-regulation of miR-9 expression was accompanied by up-regulation of CTHRC1 expression (at the mRNA and protein level). Immunohistochemistry with antibodies against CTHRC1 and S100β confirmed that CTHRC1 was expressed in Schwann cells in adult rat sciatic nerves (Fig. 3C). Collectively, we showed that the CTHRC1 expression was upregulated in Schwann cells after nerve injury. To investigate the effects of miR-9 on the expression of endogenous CTHRC1, qRT-PCR (Fig. 3D) and western blotting (Fig. 3E) were used. This showed that the expression of endogenous CTHRC1 mRNA and protein were decreased in primary Schwann cells transfected with miR-9 mimic. To determine whether CTHRC1 is regulated by miR-9 through direct binding to its 3′-UTR, the wild-type or mutant 3′-UTR of *Cthrc1* was constructed and inserted into the downstream region of the luciferase reporter gene (Fig. 3F). For luciferase activity assays, miR-9 mimic and p-Luc-UTR constructs were co-transfected into HEK 293T cells. When the wild-type 3′-UTR of *Cthrc1* was present, miR-9 induced significant reduction in the relative luciferase activity, by more than 70%. This reduction was sequence-specific because the relative luciferase activity of a UTR containing the mutant binding site did not drop as sharply as that of the UTR containing the wild-type binding site (Fig. 3G). Taken together, these results suggest that miR-9 could dramatically downregulate CTHRC1 expression by directly targeting the 3′-UTR of *Cthrc1*.

Recapitulation of the effects of miR-9 on Schwann cells by *Cthrc1* knockdown

To explore the function of CTHRC1, two specific small interfering RNAs (siRNAs) against CTHRC1 (i.e. siRNA-1 and siRNA-2) were synthesized, and both of them remarkably reduced the CTHRC1 expression at the mRNA and protein levels (Fig. 4A,B). A Transwell migration assay showed that siRNA-1 inhibited Schwann cell migration compared with control siRNA, and siRNA-2 elicited a greater inhibitory effect than siRNA-1, mainly because of a more effective knockdown of *Cthrc1* by siRNA-2 (Fig. 4C). These results indicated that knockdown of *Cthrc1* by siRNAs recapitulated the inhibitory effect of miR-9 on Schwann cells.

Because *Cthrc1* knockdown inhibited Schwann cell migration and miR-9 post-transcriptionally regulated *Cthrc1* expression by directly binding to its 3′-UTR, we hypothesized that *Cthrc1* downregulation was likely to directly mediate the miR-9-initiated Schwann cell migration. To test this hypothesis, miR-9 expression was downregulated by transfecting Schwann cells with an miR-9 inhibitor in the presence or absence of siRNA-2 against *Cthrc1*. Transfection with miR-9 inhibitor substantially increased Schwann cell migration compared with transfection with inhibitor control, whereas co-transfection with miR-9 inhibitor and siRNA-2 against *Cthrc1* significantly reversed the miR-9 inhibitor-induced increase in Schwann cell migration (Fig. 4D), suggesting that CTHRC1 is a functional mediator for miR-9.

Inhibition of Schwann cell migration by miR-9 through inactivation of Rac1 GTPase

Given that CTHRC1 is involved in modulating cell migration in various cell types (Pyagay et al., 2005; Tang et al., 2006), and that chemical inhibitors of MEK or Rac1, or dominant-negative Rac1 expression can attenuate CTHRC1-induced cell migration and adhesion (Park et al., 2013), we wanted to determine whether miR-9 regulates Schwann cell migration through inactivation of small GTPases.

The small GTPases of the Rho family, including Rac1, RhoA and Cdc42, are key regulators of cytoskeletal dynamics and cell migration. We measured the activity changes of Rac1, RhoA and Cdc42 in Schwann cells treated with *Cthrc1* siRNAs. After *Cthrc1* silencing, the relative activity of Rac1 was significantly

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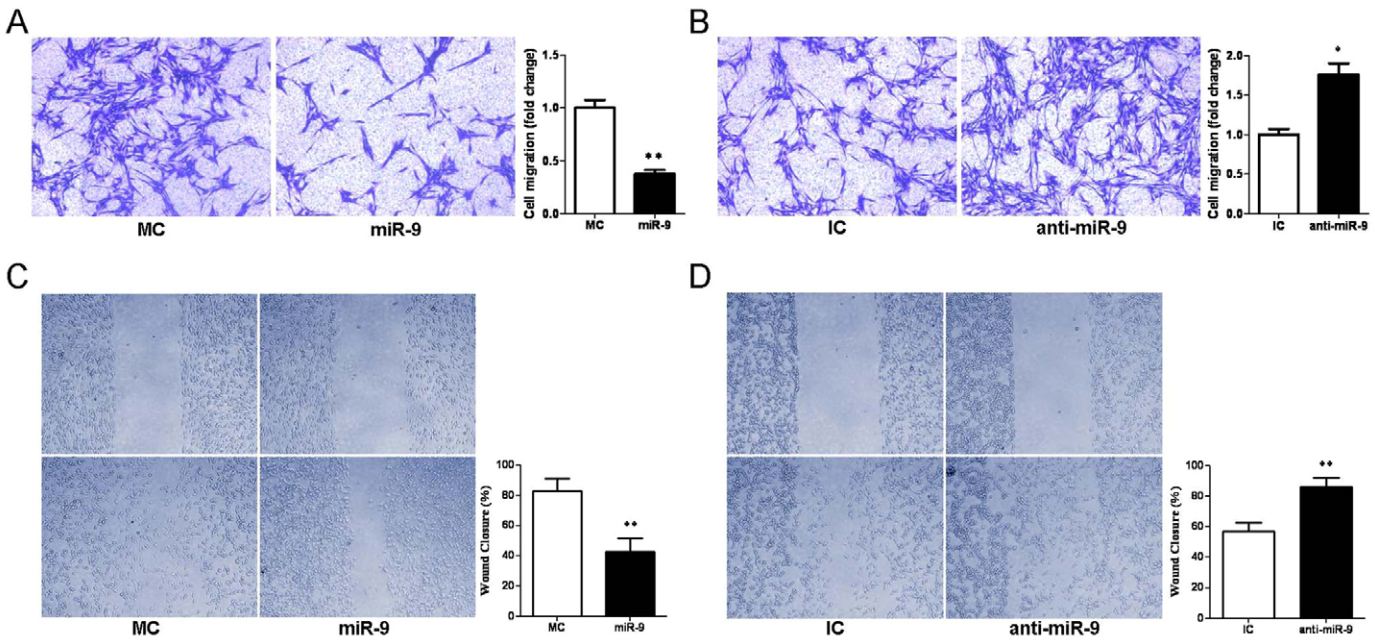

Fig. 2. Effects of miR-9 on Schwann cell migration *in vitro*. Transwell assays (A,B) and wound healing assays (C,D) both showed that cell migration of Schwann cells transfected with miR-9 mimic (miR-9) was significantly inhibited compared with that of cells transfected with mimic control (MC; A,C). Migration of Schwann cells transfected with miR-9 inhibitor (anti-miR-9) was significantly promoted compared with that of cells transfected with inhibitor control (IC; B,D). $^{**}P<0.01$, $^{*}P<0.05$.

decreased, whereas the relative activity of RhoA or Cdc42 was not decreased (Fig. 5A), suggesting that Rac1 GTPase is a downstream regulator of CTHRC1. Western blot analysis also showed that silencing of miR-9 with miR-9 inhibitor sharply elevated CTHRC1 expression, which, in turn, substantially activated Rac1 but slightly activated RhoA. Conversely, co-transfection of Schwann cells with miR-9 inhibitor and siRNA-2 against *Cthrc1* sharply reduced CTHRC1 expression, and blocked anti-miR-9-induced activation of Rac1 GTPase. In contrast, the activity of Cdc42 (another Rho GTPase) was not obviously regulated. In addition, although miR-9 inhibitor induced activation of Rac1, their total protein levels were not altered (Fig. 5B).

Since Rac1 was activated in miR-9-silenced Schwann cells, we tested whether inhibition of Rac1 kinase reversed the phenotypic changes induced by miR-9 deficiency. Migration of miR-9-silenced Schwann cells was selectively inhibited by addition of 25 μM NSC23766 (a Rac1-specific inhibitor), but there was no significant difference in cell migration between Schwann cells treated with anti-miR-9 plus NSC23766 and those treated with inhibitor control plus NSC23766 (Fig. 5C), suggesting that inhibition of Rac1 GTPase prevented the anti-miR-9-induced increase of Schwann cell migration.

Effects of miR-9 on Schwann cell migration *in vivo*

In addition to *in vitro* tests, we used an animal model of peripheral nerve transection to investigate the *in vivo* effect of miR-9 overexpression on Schwann cells within a nerve guidance conduit. In two groups of rats the sciatic nerve was transected to create a 5 mm gap between the proximal and distal stumps, and a silicone-based nerve guidance conduit was used to bridge the nerve gap (Deumens et al., 2010; Timmer et al., 2003). miR-9 agomir (steroid-conjugated miR-9 mimic) or non-targeting negative control (mixed with Matrigel) was injected into the silicone conduit. After 7 days immunocytochemistry with anti-S100β was conducted to observe Schwann cell migration from the proximal to distal stump. Both the number and the distance of Schwann cell migration was lower after injection of miR-9 agomir than after injection of non-targeting negative control (Fig. 6A). In the above experiment, miR-9 agomir or non-targeting negative control was tagged by Cy5, and the colocalization of S100β-positive and Cy5-positive cells suggested that the transfection efficiency of Schwann cells was nearly 95% (Fig. 6B). Overall, these results provided evidence that high expression of miR-9 significantly reduced Schwann cell migration *in vivo*.

DISCUSSION

In this study, we identified a new regulatory layer of the Schwann cell injury response involving post-transcriptional regulation by miRNAs. We identified miR-9 as an important functional regulator of Schwann cell migration, a crucial process of Schwann cell regenerative responses to nerve injury. *In vitro*, high expression of miR-9 inhibited Schwann cell migration whereas silencing of miR-9 enhanced Schwann cell migration. Furthermore, miR-9 might exert this regulation by targeting *Cthrc1*, which in turn inactivates downstream Rac1 GTPase. *In vivo*, high expression of miR-9 suppressed Schwann cell migration within the lumen of a silicon conduit that supported a complex regenerative microenvironment. These findings provide new evidence that miRNAs serve as important modulators for regenerative responses of Schwann cells to peripheral nerve injury.

It has been known that after nerve injury, expression of most miRNAs in Schwann cells decreases, which affects differentiation regulation of these cells (Viader et al., 2011). In this study, we found that immediately after nerve injury, eight miRNAs of Schwann cells were significantly downregulated, which might be responsible for the transcriptionally driven

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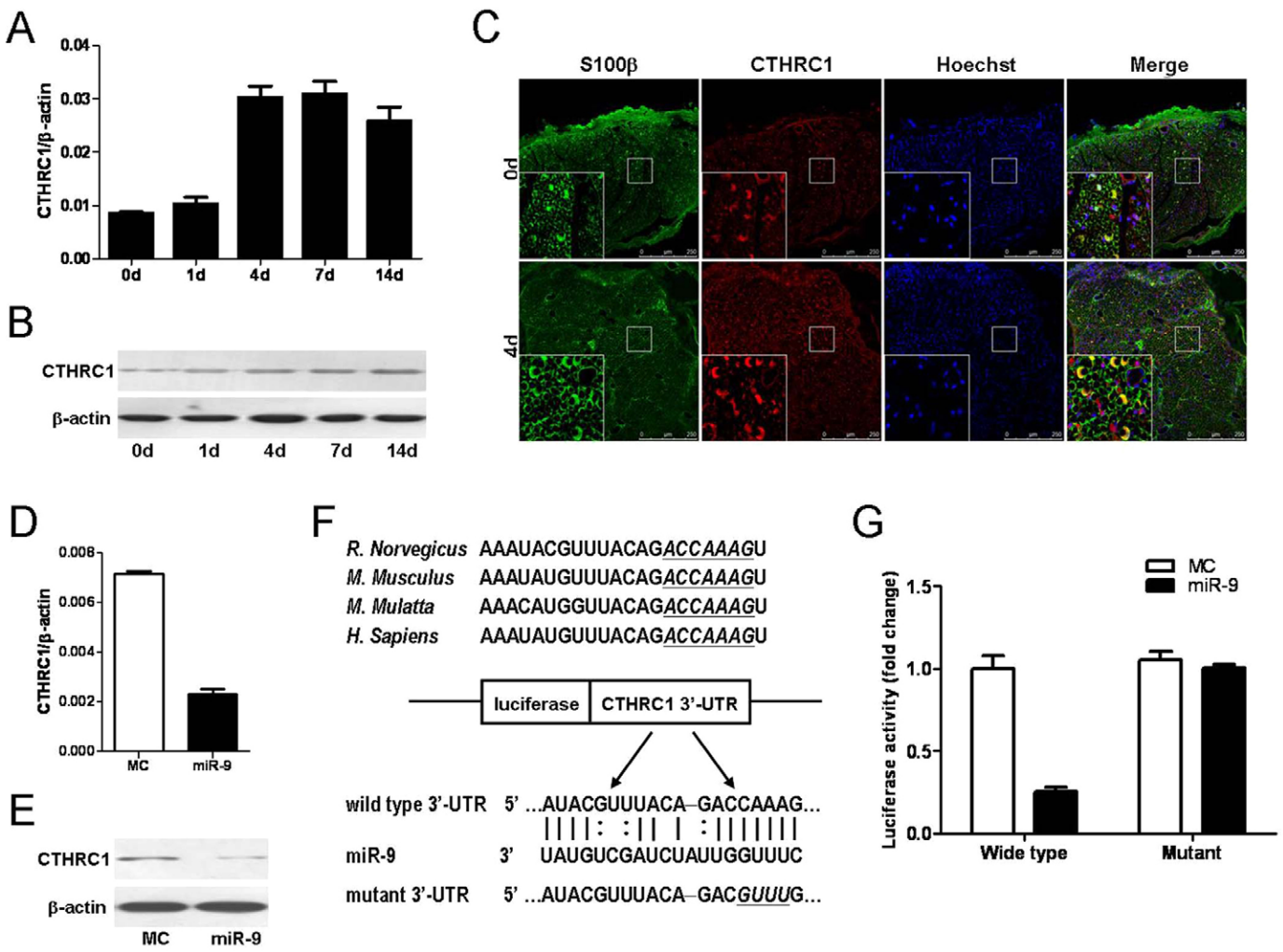

Fig. 3. miR-9-induced inhibition of CTHRC1 expression through direct targeting of its 3′-UTR. (A,B) CTHRC1 expression at the mRNA (A) and protein (B) levels was increased at 4–14 days after sciatic nerve transection compared with control (0 days). β-actin served as an internal control. (C) Transverse sections of sciatic nerves harvested at 0 days or 4 days post injury were immunostained for S100β (green) and CTHRC1 (red). Cell nuclei were counterstained with Hoechst 33342 (blue). Insets are higher magnification of the boxed areas. The merged images show that CTHRC1 is expressed in the cytoplasm of Schwann cells. (D,E) Transfection of Schwann cells with miR-9 mimic (miR-9) downregulated the expression of endogenous CTHRC1 at the mRNA (D) and protein (E) levels compared with transfection of Schwann cells with mimic control (MC). (F) Schematic showing the construction of wild-type and mutant p-Luc-UTR vectors; the mutant binding site is underlined and italicized. (G) Histogram showing the changes in the relative luciferase activity of HEK 293T cells that were co-transfected with miR-9 mimic (miR-9) or mimic control (MC) and p-Luc-UTR vectors. The *Renilla* luciferase vector was used as an internal control.

dedifferentiation of Schwann cells. GO analysis indicated that 368 potential targets of the above eight miRNAs were mainly associated with the modulation of cell localization and cell migration, rather than cell dedifferentiation and/or proliferation. And KEGG pathway enrichment analysis showed that the functions of these miRNAs were in Wnt and ErbB signaling pathways, two important pathways for molecular responses of Schwann cells after nerve injury. Wnt–planar-cell-polarity (PCP) signaling leads to the activation of the small GTPases RhoA and Rac1, which activate the stress kinase JNK and ROCK1, and lead to cytoskeleton remodeling and changes in cell adhesion and motility. Wnt–β-catenin pathway components have also been identified as essential in myelin gene expression and myelinogenesis (Tawk et al., 2011). Erbb2/3-mediated signaling is essential for Schwann cell migration and myelination (Lyons et al., 2005), and ErbB2/3 receptors, through binding to neuregulin-1, regulate the activity of the Rho GTPases family members, Cdc42 and Rac1, which can control Schwann cell migration *in vivo* (Benninger et al., 2007; Yamauchi et al., 2008). In short, injury-triggered regulation of miRNAs through their targets may be beneficial to Schwann cells involved in nerve regeneration.

In this study, we observed the expression changes of miR-9 in Schwann cells at different time points after nerve injury. miR-9 is emerging as an important regulator in development and disease because of its ability to modulate different targets in a manner dependent on the developmental stage and the cellular context (Yuva-Aydemir et al., 2011). Latest studies focus on the diverse roles of miR-9 in neuronal development and tumor formation, and indicate that miR-9 promotes neuronal differentiation and migration in several animal models (Kawahara et al., 2012; Leucht et al., 2008; Liu et al., 2012; Zhang et al., 2012). miR-9 also plays roles in proliferation and maturation of early neural progenitors cells (NPCs), and loss of miR-9 suppresses

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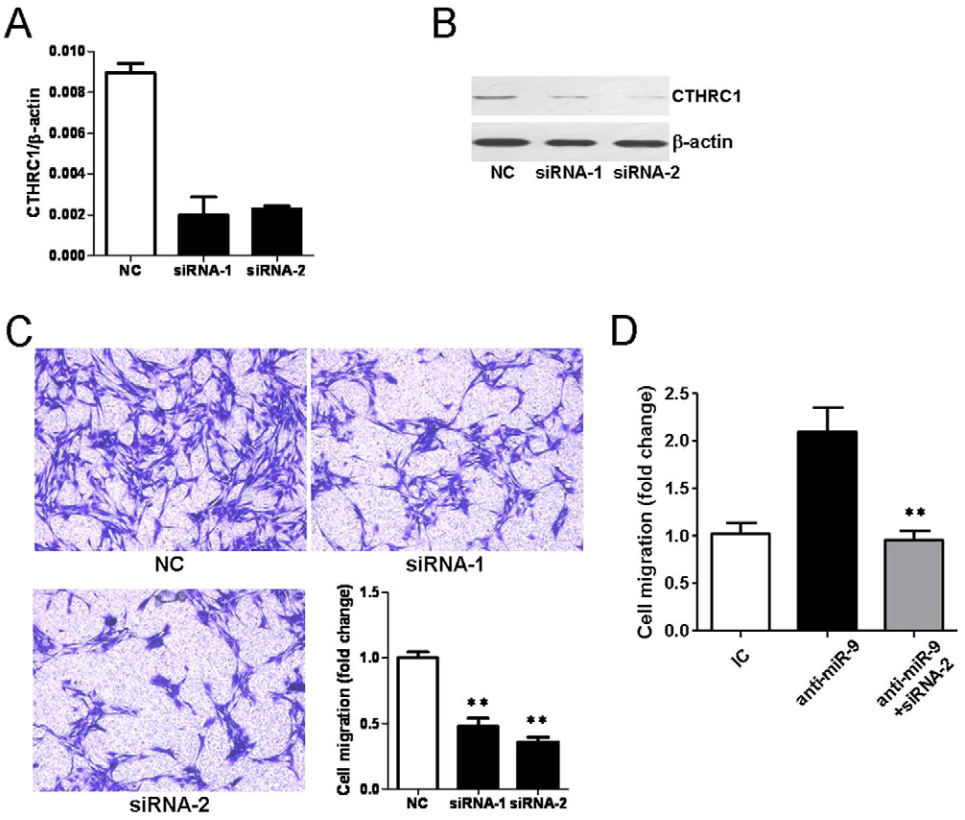

Fig. 4. Recapitulation of the effects of miR-9 by *Cthrc1* knockdown in Schwann cells. (A,B) Transfection of Schwann cells with *Cthrc1* siRNA-1 (siRNA-1) or *Cthrc1* siRNA-2 (siRNA-2) downregulated CTHRC1 expression at the mRNA (A) and protein (B) level compared with transfection with siRNA control (NC). (C) Transwell assay showing that *Cthrc1* siRNA-1 siRNA-1 or siRNA-2 significantly inhibited Schwann cell migration compared with siRNA control (NC). $^{**}P<0.01$. (D) Transwell migration assay of Schwann cells that had been transfected with miR-9 inhibitor (anti-miR-9) with or without *Cthrc1* siRNA-2. *Cthrc1* silencing reversed the promotive effect of miR-9 inhibition on Schwann cell migration. $^{**}P<0.01$.

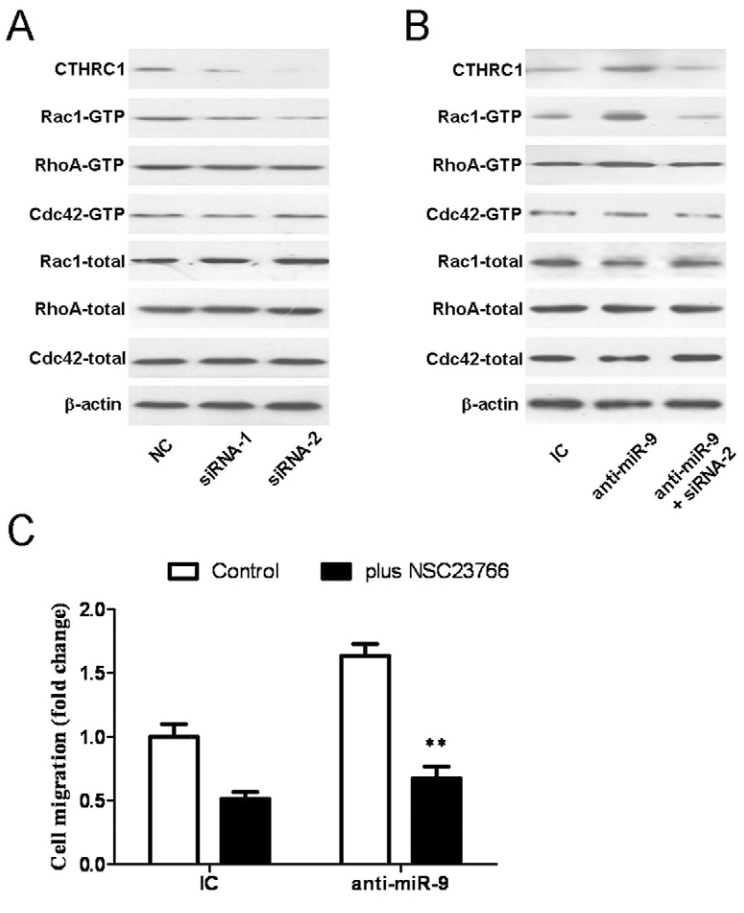

Fig. 5. miR-9-induced inhibition of Schwann cell migration through inactivation of Rac1 GTPase. (A) Western blotting showing that silencing of *Cthrc1* in Schwann cells with siRNA-1 or siRNA-2 sharply reduced CTHRC1 expression, which in turn inactivated Rac1 GTPase. (B) Western blotting showing that silencing of miR-9 in Schwann cells with miR-9 inhibitor (anti-miR-9) sharply increased CTHRC1 expression, which in turn activated Rac1 GTPase. In contrast, co-transfection with anti-miR-9 and *Cthrc1* siRNA-2 led to a sharp decrease of Rac1 GTPase compared with transfection with anti-miR-9 alone. (C) A Transwell migration assay of Schwann cells that had been transfected by miR-9 inhibitor (anti-miR-9) treated with or without Rac1 inhibitor (NSC23766). Inhibition of Rac1 GTPase reversed the promotive effect of miR-9 inhibition on Schwann cell migration. $^{**}P<0.01$.

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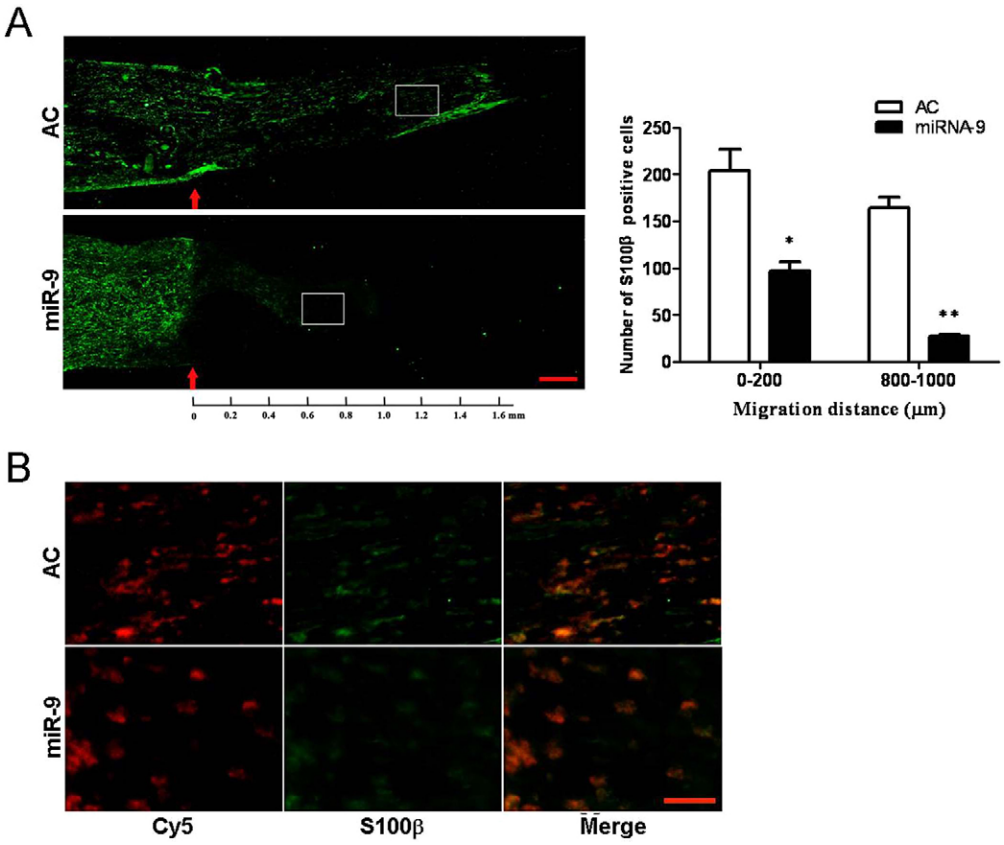

Fig. 6. Suppression of Schwann cell migration *in vivo* by increased expression of miR-9. (A) S100β immunostaining, performed at 7 days post injury, was used to determine Schwann cell migration in the regenerative conduit starting from the proximal stump of the transection site (the boarder line, indicated by red arrows). Sequential micrographs were captured and combined into a single composite image. Scale bar: 200 μm. S100β-positive (migrating) Schwann cells in the longitudinal sample section were counted between 0 and 0.2 mm or between 0.8 and 1.0 mm from the boarder line. Also shown is the comparison in the number of S100β-positive Schwann cells between animals injected with miR-9 agomir (miR-9) and agomir control (AC). **P*<0.05, ***P*<0.01. (B) Higher magnification of the boxed areas in A showing the colocalization of S100β-positive cells and Cy5-positve cells. Scale bar: 50 μm.

proliferation but promotes cell migration of cultured NPCs (Delaloy et al., 2010). In this study, we observed that miR-9 expression was downregulated in Schwann cells during a 14 day period after nerve injury, and miR-9 affected Schwann cell migration *in vitro* and *in vivo*. The downregulation trend of miR-9 was comparable with the rate of Schwann cell migration during nerve regeneration, suggesting that miR-9 downregulation triggers the phenotypic modulation of mature Schwann cells after nerve injury.

Many miRNAs exert their functions in a specific biological process mainly through a few target mRNAs. For instance, in SK-Hep-1 cells miR-9 regulates cell invasion by targeting E-cadherin, a tumor invasion suppressor (Tan et al., 2010). As another example, in melanoma cells miR-9 overexpression inhibits NF-κB1 expression, and thereby decreases cell proliferation and migration through reduction of F-actin polymerization and downregulation of multiple GTPases involved in cytoskeleton remodeling (Liu et al., 2012). In this study, we identified that CTHRC1 might be a new functional target of miR-9 during peripheral nerve injury and regeneration. *Cthrc1* has been shown to be overexpressed in injured arteries of rats, and to encode a secreted glycoprotein containing 12 repeats of a Gly-x-Tyr motif, and CTHRC1 increases cellular motility to repair the injury by promoting cell migration and limiting the deposition of collagen matrix (Pyagay et al., 2005). Aberrant expression of CTHRC1 is also observed in some metastatic solid cancers (Park et al., 2013; Tang et al., 2006; Wang et al., 2012). Therefore, CTHRC1 is considered a pro-migratory protein (Stohn et al., 2012). CTHRC1 is also a Wnt cofactor protein, and the CTHRC1 gene is located adjacent to the Frizzled (a Wnt binding receptor) gene. Cell-surface-anchored CTHRC1 can bind to Wnt, Frizzled and Ror2, and thereby form the CTHRC1–Wnt–Frizzled/Ror2 complex to enhance the activity of the non-canonical Wnt–PCP pathway, which is related to activation of small GTPases (Yamamoto et al., 2008; Wang, 2009). Previous studies have showed that the expression of dominant-negative Rac1 attenuates CTHRC1-induced cell migration and adhesion of human pancreatic cancer cells (Park et al., 2013), and that Wnt3a and Wnt5a are involved in Rac1/RhoA pathway regulation through binding to CTHRC1 (Yamamoto et al., 2008). Similar to these findings, in this study, we observed that inhibition of Rac1 GTPase reversed the promotion of Schwann cell migration by miR-9 silencing (through upregulation of CTHRC1). In future studies we aim to address the question of which specific Wnt proteins interact with CTHRC1 to promote Wnt signaling in our experimental setting.

Many growth factors, including neurotrophin-3, neuregulin-1 and insulin-like growth factor-1, stimulate migration of dedifferentiated Schwann cells (Cheng et al., 2000; Mantuano et al., 2010; Yamauchi et al., 2005). These factors promote cell migration, at least in part, by activating the Rho family GTPases. In development, Schwann cell Rac1 facilitates radial axonal sorting and myelination (Feltri et al., 2008). After nerve injury, the activity of Rac1, but not Cdc42, is increased in the injured nerves (Park and Feltri, 2011). Rac1 promotes dynamic actin remodeling, lamellipodia formation and random cell migration (Pankov et al., 2005; Petrie et al., 2009). In addition, Rac1 GTPase regulates many signaling pathways, including MAP kinases and c-Jun, which are known to regulate Schwann cell dedifferentiation. Thus, Rac1 GTPase might regulate phenotypic modulation of mature Schwann cells (Park and Feltri, 2011). Our results showed that Rac1 GTPase was obviously activated in

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Schwann cells after CTHRC1 expression was increased through silencing of miR-9. Furthermore, inhibition of Rac1 GTPase with NSC23766 reversed the promotive effect of miR-9 silencing on Schwann cell migration. Together these results suggest a new avenue for negative regulation of Wnt–PCP pathway by miR-9.

In conclusion, bioinformatics analysis identified the roles of downregulated miRNAs following peripheral nerve injury, and *in vitro* and *in vivo* tests demonstrated that Schwann cell migration was regulated by miR-9 expression. In particular, we found that miR-9 exerted its function by targeting CTHRC1 to ensure rapid and robust phenotypic modulation of Schwann cells. The effects of miR-9 on Schwann cell migration might be used to improve peripheral nerve regeneration, thus offering a new approach to peripheral nerve repair.

MATERIALS AND METHODS

Bioinformatics analysis

We collected the proximal nerve stump at 0, 1, 4, 7 and 14 days after rat sciatic nerve transection for microarray analysis. The differentially expressed miRNAs and mRNAs at successive time points were identified and the resultant expression profiles were clustering as described previously (Yu et al., 2012). Afterwards, the following analyses were carried out. (1) A search for putative targets of miRNAs in a specific profile (profile 6) with miRBase database, and integrated putative miRNA targets with differentially expressed mRNAs to yield potential targets. (2) GO and KEGG pathway enrichment analysis for the integrated targets. (3) the network for miRNAs and their targets was constructed. (4) The miRNAs and mRNAs were integrated to select the inversely correlated miRNA–target pairs, and the Pearson correlation coefficient (ρ) and the *P*-value between miRNAs and mRNAs were calculated.

qRT-PCR

Reverse-transcribed complementary DNA was synthesized with the Prime-Script RT reagent Kit (TaKaRa, Dalian, China). qRT-PCR was performed with SYBR Premix Ex Taq (TaKaRa). For miRNA detection, mature miR-9 was reverse-transcribed with a specific RT primer, quantified with a TaqMan probe, and normalized by RNU6B mature miRNA using TaqMan miRNA assays (Applied Biosystems, Foster City, CA). The relative expression level was calculated using the comparative $2^{-\Delta Ct}$ method.

In situ hybridization

In situ hybridization was performed using the miRCURY LNA™ microRNA ISH Optimization Kit (Exiqon, VedBaek, Denmark) according to the manufacturer's instructions. The nerve tissue sections were treated with 3 μg/ml proteinase K for 20 minutes at 37℃. After treatment with 0.2% glycine-PBS for 5 minutes, sections were washed twice (5 minutes each) in PBS and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine hydrochloride for 10 minutes. Hybridization with DIG-labeled probes was carried out for 2 hours at 55℃ in hybridization buffer. Afterwards, sections were washed in 5× SSC for 5 minutes at 55℃, 1× SSC twice (5 minutes each) at 55℃, 0.2× SSC twice (5 minutes each) at 55℃, and 0.2× SSC for 5 minutes at room temperature. Sections were blocked for 2 hours at room temperature with alkaline phosphatase-conjugated Fab anti-DIG antibody (Roche, Mannheim, Germany) in 2% sheep serum, followed by staining with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium (Roche) and counterstaining with Nuclear Fast Red™ (Vector Labs, Burlingame, CA).

Primary culture of Schwann cells and oligonucleotide transfection

Schwann cells were isolated from sciatic nerves of 1-day-old Sprague-Dawley rats and further treated to remove the fibroblasts using anti-Thy1.1 (Sigma, St Louis, MO) and rabbit complement (Invitrogen, Carlsbad, CA) as described previously (Mantuano et al., 2008). The final cell preparation consisted of 98% Schwann cells, as determined by immunostaining with anti-S100β, a specific marker of Schwann cells. Primary culture of Schwann cells was maintained in DMEM containing 10% fetal bovine serum (complete medium; Invitrogen) at 37℃ under humidified 5% CO_2. The cultured Schwann cells were passaged no more than three times prior to use.

Schwann cells were transfected with miRNA mimic, miRNA inhibitor or siRNAs (Ribobio, Guangzhou, China), respectively, using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions. The sequences of siRNA duplexes are listed in supplementary material Table S5.

Transwell migration assay

Schwann cells were examined using 6.5 mm Transwell chambers with 8 μm pores (Costar, Cambridge, MA) as described previously (Mantuano et al., 2008). The bottom surface of each membrane was coated with 10 μg/ml fibronectin. A 100 μl sample of DMEM containing resuspended Schwann cells (10^6 cells/ml) was transferred to the top chamber of the Transwells and the cells were allowed to migrate at 37℃ in 5% CO_2 before addition of 600 μl complete medium into the lower chambers. At specified times, the upper surface of each membrane was cleaned with a cotton swab. Cells adhering to the bottom surface of each membrane were stained with 0.1% Crystal Violet, imaged, and counted using a DMR inverted microscope (Leica Microsystems, Bensheim, Germany). Assays were performed in triplicate using three wells each.

To determine the effect of Rac1 inhibitor on Schwann cell migration, Schwann cells transfected with miRNA inhibitor for 36 hours were treated with 25 μM NSC23766 (Merck Millipore, Darmstadt, Germany) for 3 hours. The cells were then subjected to a Transwell assay.

Wound healing assay

The wound healing assay was performed using Culture-Insert (Ibidi, Martinsried, Germany) as previously described (Takai et al., 2012; Vetrano et al., 2011). The culture insert was placed into one well of the 12-well plate and slightly pressed on the top to ensure tight adhesion. 3.5×10^4 Schwann cells transfected with miRNA mimic or miRNA inhibitor were seeded to each well and incubated for 24 hours. The confluent monolayers of cells were starved in DMEM supplemented with 0.5% fetal bovine serum (Invitrogen) and 0.15 μg/ml mitomycin C (Sigma) for 12 hours. Afterwards, the insert was removed with tweezers yielding a standardized wound of 500 μm. The dish was washed and subsequently imaged under the above medium for 10 hours. Closure of the wound was monitored and photographed at multiple sites, and representative images were captured. The images were subjected to Wimscratch Quantitative Wound Healing Image Analysis (Wimasis GmbH, Munich, Germany).

Cell viability assay

For the cell viability assay, viable cells were counted using Cell Counting Kit-8 from Dojindo (Kumamoto, Japan). Schwann cells were transfected with miRNA mimic or miRNA inhibitor. After replacing the medium with 100 μl DMEM containing 10% fetal bovine serum, 10 μl CCK-8 was added at the indicated time points, and plates were incubated for an additional 2.5 hours. The absorbance at 450 nm was recorded to determine the number of viable cells in each well.

Cell proliferation assay

Schwann cells were plated at a density of 2×10^5 cells/ml onto 0.01% poly-L-lysine-coated 96-well plates. At the indicated time points after cell transfection, 50 μM EdU was applied to the cell culture which was then incubated for an additional 2 hours. The cells were fixed with 4% formaldehyde in PBS for 30 minutes. After labeling, the cells were analyzed using a Cell-Light EdU DNA Cell Proliferation Kit (Ribobio) according to the manufacturer's protocol. Schwann cell proliferation was expressed as the ratio of EdU-positive cells to total cells, which was determined using images of randomly selected fields obtained on a DMR

fluorescence microscope (Leica Microsystems). Assays were performed in triplicate using three wells each.

Western blot analysis and Rho GTPases activation assay

Protein extracts were prepared from cell cultures or proximal nerve stumps. Equal amounts of protein were subjected to SDS-PAGE and electrotransferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% nonfat dry milk in Tris-HCl-buffered saline, pH 7.4 with Tween 20, and incubated with the primary antibody against CTHRC1 (Proteintech Group, Chicago, IL) according to the manufacturer's recommendations. Antibody binding was detected with an HRP-conjugated species-specific secondary antibody followed by an enhanced chemiluminescence assay (Pierce Chemical Company, Rockford, Illinois).

The activation of the Rho family GTPases was determined with Rho and Rac1/Cdc42 activation assay kits (Upstate Biotechnology, Lake Placid, NY) in accordance with the manufacturer's instructions. Analysis was performed by SDS-PAGE and western blotting with anti-RhoA (1:2000), anti-Rac1 (1:2000) and anti-Cdc42 (1:2000) antibodies.

Immunochistochemistry

The proximal nerve stump was harvested at 0 and 4 days after sciatic nerve transaction. The nerve samples were fixed with 4% paraformaldehyde and dehydrated in sucrose solution. Cryostat sections of 12 μm were cut and placed on slides that were frozen at −80℃. For staining, the slides were thawed and rinsed in PBS, followed by permeabilization for 1 hour in 5% goat serum, 1% BSA, and 0.3% Triton X-100. Then, anti-CTHRC1 antibody (1:100; Proteintech Group) or anti-S100β antibody (1:200, Sigma) was incubated with the slides at 4℃ for 12 hours. After rinsing, the slides were reacted with Alexa Fluor 488 goat anti-mouse IgG (H^+L; 1:1000; Invitrogen) and Cy3 sheep anti-rabbit IgG (1:400; Sigma) at room temperature for 2 hours. The slides were counterstained with Hoechst 33342 for 5 minutes. All samples were visualized under a confocal microscope (Leica Microsystems).

Luciferase reporter assay

The 3′-UTR sequence of *Cthrc1* was amplified from the genomic DNA and subcloned into the region directly downstream of the stop codon of the luciferase gene in the luciferase reporter vector. With appropriate primers, PCR amplification of the 3′-UTR sequence of *Cthrc1* generated different p-Luc-UTR luciferase reporter vectors. The sequences of wild-type and mutant 3′-UTR were confirmed by sequencing. HEK 293T cells were seeded in 96-well plates and transfected with a mixture of 30 ng p-Luc-UTR, 5 pmol miRNA mimic, and 5 ng *Renilla* luciferase according to the recommended protocol for the Lipofectamine 2000 transfection system (Invitrogen). After 48 hours incubation, the activity of firefly and *Renilla* luciferases was measured from the cell lysates using the dual-luciferase reporter assay system (Promega, Madison, WI).

In vivo Schwann cell migration

Adult, male Sprague-Dawley rats were anaesthetized by an intraperitoneal injection of complex narcotics. Following skin incision, the left sciatic nerve was exposed and resected to leave a nerve gap between the proximal and distal stumps. A silicone conduit (1.0 mm internal diameter) was used to bridge the nerve gap with the proximal nerve stump anastomosed to the conduit at the junction. After silicone conduits had been inserted into the nerve gap in two groups of rats ($n=3$ each group), a mixture (1:1, volume ratio) of Matrigel (BD Biosciences, Billerica, MA) and steroid-conjugated miR-9 (also called miR-9 agomir) or control agomir (Ribobio), respectively, was injected into the conduit lumen using a pre-cooled micropipette from the opposite opening of the conduit, followed by anastomosis of the conduit to the distal nerve stump. The injection was done as slowly as possible to prevent the formation of air bubbles. Afterwards, the surgical incision was closed in a routine fashion, and animals were housed in large cages with sawdust bedding to minimize the discomfort and possible painful mechanical stimulation. All the experimental procedures involving animals were conducted in accordance with institutional animal care guidelines and approved ethically by the Administration Committee of Experimental Animals, Jiangsu Province, China.

At 7 days after surgery, rats were killed and the silicon conduits containing regenerated nerves were harvested for sectioning. Immunohistochemistry was performed in the sections using anti-S100β as per standard protocols. The edge of the proximal nerve stump was identified and labeled. S100β-positive cells were identified at 0.2 mm intervals from the proximal nerve stump toward the distal nerve stump, and the cell displacement and cell number were measured, which served as the parameters for characterizing Schwann cell migration during nerve regeneration.

Statistical analysis

Statistical analyses were performed using SPSS 15.0 for windows (SPSS, Chicago, IL). Student's *t*-test was used for comparison between groups. $P<0.05$ was considered statistically significant. All data are expressed as means ± s.d.

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Competing interests

The authors declare no competing interests.

Author contributions

S.Z., R.G., T.Q. and N.W. planned and carried out the experiments. S.Z. and B.Y. wrote and edited the mansucript and figures. W.H. contributed to *in vivo* migration studies. G.D. contributed to data analysis. F.D., B.Y. and X.G. contributed to study design and management, and manuscript production.

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Supplementary material

Supplementary material available online at
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