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Original Research Article

MiR-126 ameliorates neuropathic pain through suppression of macrophage migration and inflammatory response by targeting EFhd2

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Abstract

Purpose: *To study the molecular mechanism of miR-126 in partial sciatic nerve ligation-induced neuropathic pain.*
Methods: *Mice were divided into four groups: sham, partial sciatic nerve ligation (PSL), PSL+miR-negative control (NC) agomir, and PSL+miR-126 agomir. Tactile allodynia and thermal hyperalgesia were tested to evaluate the effect of PSL surgery and miR-126 overexpression on mice in each group. Expression levels of TNF-α, IL-6, and IL-1β were tested by enzyme-linked immunoassay to verify the anti-inflammatory effects of PSL surgery and miR-126 overexpression. Immunofluorescence staining was applied to investigate macrophage number, whereas quantitative PCR was applied to investigate miR-126 expression levels in each group. J774A.1 macrophage migration was evaluated by Transwell assays after administration of lipopolysaccharide (LPS) and transfection with miR-126 mimics and NC mimics, respectively. Finally, bioinformatics methods and dual-luciferase reporter assays were applied together to ascertain the interaction between miR-126 and EFhd2.*
Results: *MiR-126 expression was downregulated in the sciatic nerve of neuropathic pain model mice, and miR-126 overexpression alleviated neuropathic pain symptoms. Overexpression of miR-126 inhibited the inflammatory response and migration of macrophages in mice. miR-126 targets and regulates EFhd2 expression level.*
Conclusion: *MiR-126 suppresses macrophage inflammatory response and migration, and ameliorates PSL-induced neuropathic pain in mice by targeting EFhd2.*

Keywords: *EF-hand domain-containing protein D2, MicroRNA-126, Macrophage, Neuropathic pain, Partial sciatic nerve ligation*

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INTRODUCTION

Neuropathic pain is a pain syndrome induced by primary pathological changes or dysfunction of the central or peripheral nervous system or peripheral nerve, posterior spinal root, spinal cord, and central nerve injury [1]. Neuropathic pain has a great impact on the life of patients, and current treatment methods are very limited [2]. Macrophages and microglia are the main inflammatory cells involved in spinal cord injury. Current studies have identified two phenotypes

of macrophages, including M1 and M2 macrophages, and spinal cord injury promoted the polarization of macrophages to M1 type [3]. M1 macrophages synthesize and release many inflammatory cytokines and proteolytic enzymes, thus causing neurotoxic effects [4]. Some research has found that adjusting the phenotype shift of macrophage polarity from M1 to M2 after central nervous system injury promoted nerve injury repair.

Recent studies have shown that expression of microRNAs in the nervous system regulated neuroinflammation and neuropathic pain [5]. Some works have found that miR-126 expression is downregulated in rat serum and brain tissue after undergoing spinal nerve ligation surgery or ischemia [6]. MiR-126 secreted by exosomes inhibits activation of microglia and secretion of inflammatory cytokines [7]. MiR-126 also plays a role in vascular cognitive impairment, neuronal protection, and inhibition of glioma migration and invasion [8]. However, there has been no study on the role of miR-126 in neuropathic pain and the underlying mechanism.

Expression of EF-hand domain-containing protein D2 (EFHD2) can promote neurodegenerative diseases [9] and stimulate macrophage migration [10], whereas EFHD2 knockdown inhibited macrophage production of interferon gamma, interleukin (IL)-10, tumor necrosis factor alpha (TNF-α), IL-6, and IL-1β [11]. EFhd2 is a conserved calcium-binding protein that has been linked to various neurological diseases and cancer and is a target of miR-126 [12].Therefore, EFHD2 may participate in macrophage-induced inflammation and neuropathic pain. Here, the role of miR-126 was investigated, as well as its potential target in a mouse model of neuropathic pain, which may provide a new target for treating neuropathic pain.

EXPERIMENTAL

Animals and establishment of the PSL mouse model

Male ICR mice (4 weeks old) were obtained from the Institute of Laboratory Animal Sciences, Cams & Pumc (Beijing, China). All experimental procedures and animal welfare were performed according to the National Institutes of Health Laboratory Animal Care and Use Guidelines [13] and were approved by the Animal Ethics Committee of the Third People's Hospital of Gansu Province (approval no.2019-032). All mice were provided with adequate food, clean water, and an appropriate habitat, free of disease and fear.

Male ICR mice were divided into four groups (n = 6): sham, PSL, PSL+miR-NC agomir, and PSL+miR-126 agomir group. Male ICR mice in the three PSL-treated groups underwent surgery to produce PSL and induce neuropathic pain according to the protocol [14]. Male ICR mice in the PSL+miR-NC agomir or PSL+miR-126 agomir group were subjected to Matrigel (Corning, NY, USA) injection. A mixture of Matrigel and steroid-conjugated miR-126 (1:1 v/v) or agomir control were injected into the sciatic nerve gap. The incision was then closed using the standard procedure and the animals were placed in large cages fitted with sawdust sheets to reduce discomfort and pain from possible mechanical stimuli. No animals died accidentally during the experiment.

Determination of microRNA overexpression in male PSL mouse model

To overexpress microRNA in male PSL mice, ICR mice in the PSL+miR-NC agomir group and PSL+miR-126 agomir group underwent surgery. Briefly, a 1-mm silicone conduit was used to bridge the 4-mm sciatic nerve gap between the distal and proximal stumps, and a mixture of Matrigel and steroid-conjugated miR-126 (miR-126 agomir, Ribobio, Guangzhou, China) (1:1 v/v) or agomir control (miR-NC agomir, Ribobio) was injected into the conduits using a micropipette. The injection process was slow to prevent bubble formation. The incision was then closed using the standard procedure, and the animal was placed in a large cage fitted with sawdust flakes to reduce discomfort and pain from possible mechanical stimuli.

Animal behavioral tests

Tactile allodynia was tested with an electrical von Frey filament (IITC Life Science, Woodland Hills, CA, USA), whereas thermal hyperalgesia was separately detected using Hargreaves tests according to a previously published protocol [14]. In the von Frey test, mice were put on a 6 × 6-mm wire mesh floor covered with an opaque cup. After two to three hours of adjustment, 0.07 g of von Frey filaments (Neuroscience, Japan) were used to stimulate the middle of the hind paw through the bottom of the mesh floor. Withdrawal responses of each hind paw were measured 10 times, and the number of withdrawal responses to stimulation was recorded to determine the tactile allodynia. For the Hargreaves test, mice were put on a glass panel and covered using a transparent cage. After two to three hours of

adjustment, a radiant heat source (IITC 390 Plantar Test Analgesia Meter, Neuroscience) was placed under the glass panel and used to stimulate the plantar surface of the hind paw. Withdrawal latencies of each hind paw were measured three times, and the average latency of three stimulations was calculated to evaluate thermal hyperalgesia. After the behavioral test, the mice in each group were sacrificed by cervical dislocation under anesthesia with isofluorane inhalation.

Cell culture

Mouse macrophage line J774A.1 was obtained from the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Gibco DMEM culture medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10 % Gibco fetal bovine serum (FBS; Thermo Fisher Scientific) and 1 % Gibco penicillin/streptomycin (Thermo Fisher Scientific) in a humidified 5 % CO_2 incubator. In order to induce a macrophage inflammatory response, cells were treated with 50 ng/mL lipopolysaccharide (LPS) for 24 h.

Quantitative polymerase chain reaction (qPCR)

Cells were first treated using Trizol reagent (Invitrogen, Thermo Fisher Scientific) to extract total RNA. Expression of miR-126, IL-1β, IL-6, TNF-α, and EFhd2 was detected via qPCR using SYBR Premix EX Taq (Takara, Japan). Expression levels of miRNA and mRNA were normalized using U6 small nuclear RNA and β-actin. Expression levels of miR-126, IL-1β, IL-6, TNF-α, and EFhd2 in every group were measured using the $2^{-\triangle\triangle Ct}$ method. Primer sequences are listed in Table 1.

Enzyme-linked immunosorbent assay (ELISA)

Expression levels of IL-6 (ab100712; Abcam, Cambridge, UK), IL-1β (ab100705; Abcam), and TNF-α (ab100747; Abcam) in the sciatic nerve of male PSL mice and J774A.1 macrophages were determined using the corresponding ELISA kits according to the manufacturer's instructions.

Immunofluorescence staining

Sciatic nerve tissues were fixed with 4 % paraformaldehyde for 12 min and permeabilized in 0.2 % Triton X-100 for 30 min. Tissues were then blocked with 5 % bovine serum albumin for 0.5 h and incubated with primary antibody CD68 (rat anti-CD68 antibody, ab125212, 1:1,200; Abcam) overnight at 4 °C. Finally, the tissues were incubated with secondary antibody (Abcam) for 2 h followed by counterstaining with DAPI.

Table 1: Primers for miR-126, U6, TNF-α, IL-6, IL-1β, EFhd2 and reference genes

Gene	Primer	Sequence (5'→3')
miR-126	Forward	GCCGACGAACCGAGAGAAAAAA
	Reverse	GTGCAGGGTCCGAGGT
U6	Forward	TCCTCCACGACAACCAAAACC
	Reverse	TCTTTTCCCAAAATCCCAGACTC
TNF-α	Forward	GCCACCACGCTCTTCTGTCTAC
	Reverse	GGGTCTGGGCCATAGAACTGAT
IL-6	Forward	CACATGTTCTCTGGGAAATCG
	Reverse	TTGTATCTCTGGAAGTTTCAGATTGTT
IL-1β	Forward	ACCTTCCAGGATGAGGACATGA
	Reverse	CTAATGGGAACGTCACACACCA
EFhd2	Forward	TCCGGGAGTTCCTCCTGATT
	Reverse	AAGCTCTTCGCTCCCTTGAC
β-actin	Forward	GTGACGTTGACATCCGTAAAGA
	Reverse	GCCGGACTCATCGTACTCC

Transwell assay

Cells (3.0×10^4) were seeded in Matrigel-precoated transwell inserts (Costar, Manassas, VA, USA) and cultured under different conditions. Normal medium and serum-free medium were separately added to the lower chamber and upper chamber, respectively. Cells migrated to the lower surface of the membrane after one day. The cells were fixed in 4 % paraformaldehyde for 15 min and stained with 0.1 % crystal violet solution for 30 min. Cells were counted after drying the culture plate.

Cell transfection

Synthetic miR-126 mimics and NC mimics were acquired from GenePharma (Shanghai, China). Cells were cultured in 6-well plates for one day, and Lipofectamine 2000 (Thermo Fisher Scientific) was used to transfect HEK293 cells with the miR-126 mimics and NC mimics.

Dual-luciferase reporter assay

EFhd2-WT and EFhd2-MUT were separately constructed by cloning the fragments of EFhd2 mRNA wild-type or mutant-type binding sites, respectively, of miR-126 into pmirGLO luciferase

reporter vector (Promega, Madison, WI, USA). Then, miR-126 mimics or NC mimics were co-transfected with reporter plasmids into HEK293 cells, and luciferase activity was measured using the Dual-Luciferase kit (Promega) after 48 h.

Statistical analysis

All data are expressed as mean ± standard error of the mean, n = 3. Comparison of differences between two groups was performed using one-way ANOVA. *P* value of < 0.01 (two-tailed) was considered statistically significant.

RESULTS

Expression of miR-126 was downregulated in the sciatic nerve of the neuropathic pain mouse model

The animal PSL model was established to induce neuropathic pain, and then animal behavioral tests were performed to observe the behavioral performance of mice that had undergone PSL surgery. The results revealed that tactile allodynia appeared, whereas thermal hyperalgesia disappeared, after PSL surgery (*p* < 0.01) (Figure 1 A and B). Appearance of tactile allodynia and disappearance of thermal hyperalgesia indicate that the animal pain model has been successfully established. Then, ELISA assays were conducted to investigate the inflammatory cytokines in the sciatic nerve. The results suggested that IL-6, IL-1β, and TNF-α were significantly increased in the PSL group, indicating that PSL surgery elicited an inflammatory response (*p* < 0.01) (Figure 2 A). Subsequently, immunofluorescence staining of sciatic nerve tissue was used to label macrophages. The results revealed that more CD68-positive macrophages were observed in the sciatic nerve in the PSL group than in the sham group, which further indicated that PSL surgery induced an inflammatory response (*p* < 0.01) (Figure 2 B). Moreover, qPCR results revealed that expression of miR-126 RNA was downregulated in the sciatic nerve in the mouse model of neuropathic pain (*p* < 0.01) (Figure 2 C).

Overexpression of miR-126 alleviated neuropathic pain symptoms in mice

The qPCR results showed that miR-126 expression was downregulated and upregulated in the PSL+miR-NC agomir group and PSL+miR-126 agomir group, respectively, indicating that miR-126 was successfully overexpressed in male PSL mice (*p* < 0.01) (Figure 4 C).

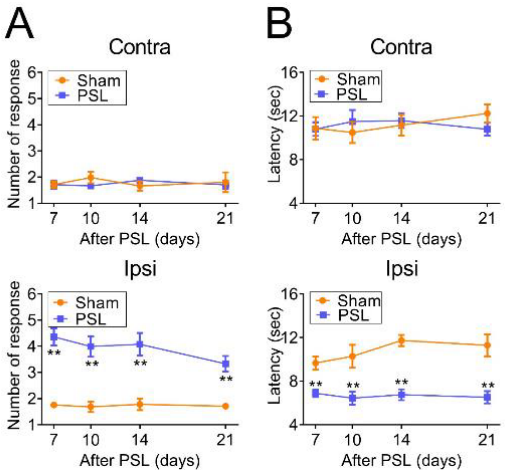

Figure 1: Establishment of an animal pain model. (A) Tactile allodynia and (B) thermal hyperalgesia were assessed by the von Frey test and Hargreaves test, respectively. Ipsi, ligation of the ipsilateral sciatic nerve; contra, ligation of the contralateral sciatic nerve. ***P* < 0.01 indicates significant difference versus sham group

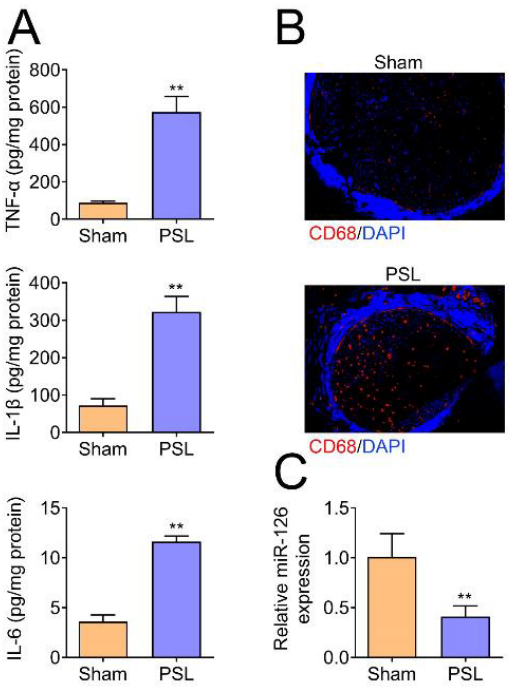

Figure 2: miR-126 expression was downregulated in the sciatic nerve in a mouse model of neuropathic pain. (A) The production of inflammatory cytokines, including TNF-α, IL-6, and IL-1β. (B) Protein expression levels of CD68 by fluorescence microscopy. (C) Expression level of miR-126. ***P* < 0.01 indicates significant difference between the two groups.

Then, animal behavioral tests revealed that tactile allodynia and thermal hyperalgesia of mice appeared and disappeared in the PSL+miR-NC

agomir group, respectively, whereas both were alleviated in the PSL+miR-126 agomir group (*p* < 0.01) (Figure 3 A and B). ELISA results suggested that expression of IL-6, IL-1β, and TNF-α was increased in the PSL+miR-NC agomir group but markedly decreased in the PSL+miR-126 agomir group (*p* < 0.01), indicating that miR-126 relieves the inflammatory response (Figure 4 A). Moreover, immunofluorescence staining of sciatic nerve tissues showed that more CD68-positive macrophages accumulated in the PSL+ miR-NC agomir group than in the sham group. However, the number of CD68-positive macrophages was decreased in the PSL+miR-126 agomir group (*p* < 0.01), suggesting that miR-126 reduced the inflammatory response (Figure 4 B). These results suggest that overexpression of miR-126 alleviated neuropathic pain symptoms in mice.

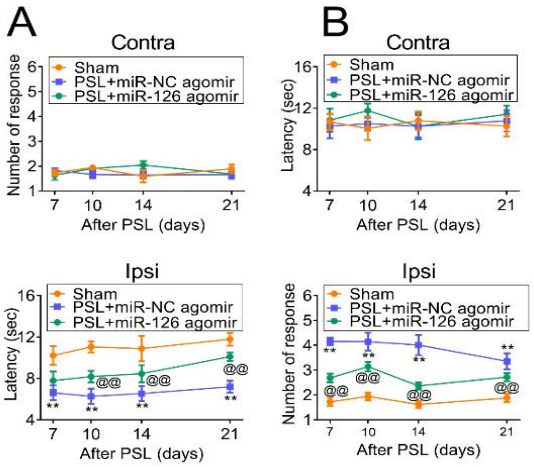

Figure 3: Overexpression of miR-126 alleviates neuropathic pain symptoms in mice. (A) Tactile allodynia and (B) thermal hyperalgesia assessed in PSL mice injected with or without miR-126 agomir and the sham group. ***p* < 0.01 indicates significant difference versus sham group, and @@*p* < 0.01 indicates significant difference versus PSL+miR-NC agomir group

Overexpression of miR-126 inhibited the inflammatory response and migration of macrophages in mice

To explore the effect of miR-126 on the inflammatory response and migration of macrophages *in vitro*, mouse J774A.1 macrophages were administered LPS (50 ng/mL) for 24 h and then transfected with miR-126 mimics and NC mimics. Next, the protein and mRNA expression levels of IL-6, IL-1β, and TNF-α in J774A.1 macrophages were detected. Both ELISA and qPCR results showed that LPS treatment increased IL-6, IL-1β, and TNF-α in macrophages (*p* < 0.01). However, overexpression of miR-126 markedly reduced the LPS-induced increase in inflammatory cytokine (*p* < 0.01) (Figure 5 A and B). Furthermore, transwell assay results revealed that LPS administration increased the number of invaded J774A.1 macrophages. However, the effect of LPS treatment was effectively counteracted by the overexpression of miR-126 (*p* < 0.01) (Figure 5 C). These results revealed that miR-126 inhibits the inflammatory response and the migration of macrophages.

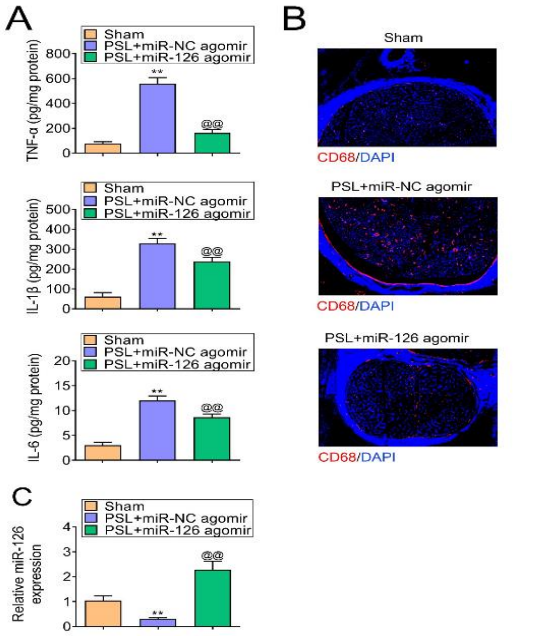

Figure 4: Overexpression of miR-126 relieves the inflammatory response. (A) Inflammatory cytokines, including TNF-α, IL-6, and IL-1β, in PSL mice injected with or without miR-126 agomir and the sham group. (B) The protein expression of CD68 in the sciatic nerve in PSL mice injected with or without miR-126 agomir and sham group. (C) Expression of miR-126 in the sciatic nerve in PSL mice injected with or without miR-126 agomir and the sham group. ***p* < 0.01 indicates significant difference versus sham group, and @@*p* < 0.01 indicates the significant difference versus PSL + miR-NC agomir group

MiR-126 targeted and regulated the expression of EFhd2

To study the mechanism of miR-126 regulation of J774A.1, the mRNA binding sites were predicted with TargetScan. The results showed that EFhd2 mRNA was a binding target of miR-126. The anticipated 3'-UTRs of EFhd2 mRNA binding to miR-126 are presented in Figure 6 A. In order to verify whether EFhd2 was a potential target of miR-126, EFhd2 WT and MUT fragments were cloned downstream of the firefly luciferase coding region.

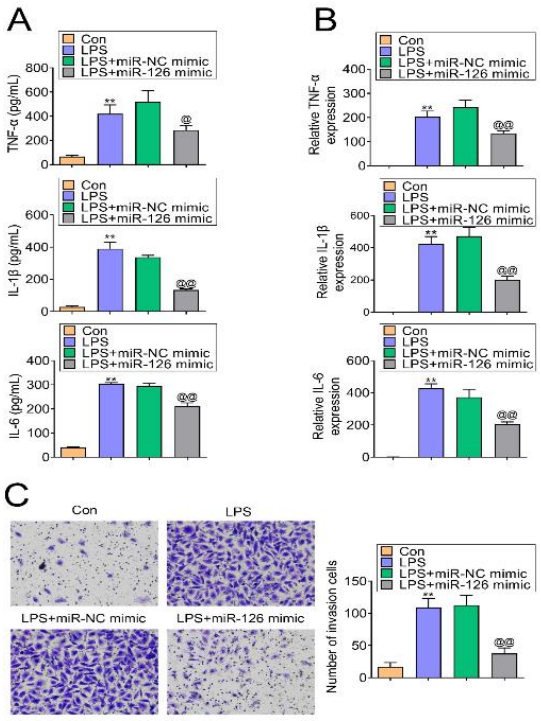

Figure 5: Overexpression of miR-126 inhibited the inflammatory response and migration of macrophages in mice. (A) Protein expression of TNF-α, IL-6, and IL-1β in J774A.1 macrophages transfected with miR-126 mimics and NC mimics (control) after administration of lipopolysaccharide (LPS) (50 ng/mL) for 24 h. (B) mRNA expression levels of TNF-α, IL-6, and IL-1β in J774A.1 macrophages transfected with miR-126 mimics and NC mimics (control) after administration of LPS (50 ng/mL) for 24 h. (C) Transwell assay of J774A.1 in each group. $^{**}p < 0.01$ indicates significant difference versus sham group, $^{@}p < 0.05$; $^{@@}p < 0.01$ indicates the significant difference versus PSL+miR-NC agomir group

The results showed that overexpression of miR-126 markedly lowered the luciferase activity of the EFhd2-WT reporter gene, but not that of the EFhd2-MUT control **(**$p < 0.01$**) (**Figure 6 B**)**. To further study whether miR-126 regulated EFhd2 expression, the expression of EFhd2 mRNA and protein was tested in J774A.1 cells transfected with miR-NC mimics, miR-126 mimics, miR-NC inhibitor, and miR-126 inhibitor. The qPCR and western blot assay results revealed that EFhd2 protein expression was, as expected, upregulated in the miR-126 inhibitor-transfected group **(**$p < 0.01$**)**. Whereas, compared with NC mimics, miR-126 mimics significantly inhibited EFhd2 protein expression **(**$p < 0.01$**) (**Figure 6 C and D**)**. These data demonstrated that EFhd2 is a target of miR-126, and miR-126 repressed EFhd2 expression at both the mRNA and protein levels.

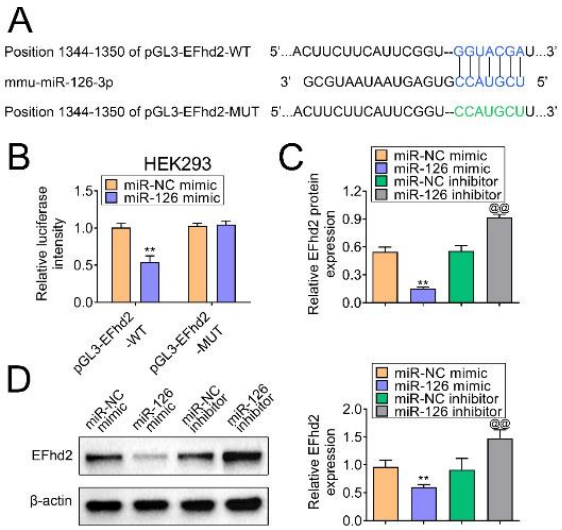

Figure 6: MiR-126 can target and regulate EFhd2 expression. (A) Prediction of miR-126 binding sites on target gene EFhd2 by TargetScan. (B) Dual-luciferase reporter cell signal intensity in cells co-transfected with EFhd2-WT or EFhd2-MUT and miR-126 mimics and NC mimics. (C and D) mRNA and protein expression levels of EFhd2 in cells as determined using qPCR and western blot. $^{**}p < 0.01$ indicates significant difference versus miR-NC mimics group, and $^{@@}p < 0.01$ indicates the significant difference versus miR-NC inhibitor group

DISCUSSION

Recently, some studies have shown that PSL can be used as a neuropathic pain model. Macrophages and microglia are the main inflammatory cells present after PSL surgery. Extracellular mechanical and intracellular molecular signals lead to the pathological changes in macrophages. However, the molecular regulatory mechanism that determines the fate of macrophages during PSL-induced neuropathic pain remains poorly understood. Therefore, it is necessary to elucidate the underlying molecular mechanism that regulates the activity of macrophages during the PSL-induced neuropathic pain, which may suggest new therapeutic targets for treating neuropathic pain. MiRNAs have been reported to play crucial roles in regulating cell migration and the inflammatory response [15,16]. Many miRNAs are highly expressed in the central or peripheral nervous system, contributing to the maintenance of normal tissue homeostasis and functions [17]. Moreover, many miRNAs are involved in the inflammatory response and migration of macrophages, and aberrant expression of miRNAs was observed in PSL. Specifically, miR-150 has been reported to restrain neuropathic pain in an AKT3-dependent pathway [18]. In addition, miR-194 alleviates neuropathic pain

and inhibits neuro-inflammation through targeting FOXA1 [19].

Moreover, miR-140 has been found to attenuate neuropathic pain via targeting S1PR1 [20]. However, the specific molecular mechanisms by which miRNAs regulate the inflammatory response of macrophages and their migration involved in PSL need to be further investigated. This study revealed that miR-126 expression was downregulated in the sciatic nerve of a mouse model of neuropathic pain. Overexpression of miR-126 suppressed the migration and inflammatory response of macrophages and alleviated the neuropathic pain symptoms in mice by regulating macrophage polarity from M1 to M2 phenotype. These results indicate that miR-126 may be an inhibitor of the inflammatory response of macrophages and their migration in PSL.

A growing number of studies have shown that miRNAs serve their functions by regulating the expression of targeted mRNAs [21]. For example, upregulation of miR-154-5p could decrease neuropathic pain via negatively regulating the lncRNA MALAT1/AQP9 signaling pathway [22], whereas miR-129-5p participates in protecting the nervous system via targeting the HMGB1 signaling pathway in rats [23]. Furthermore, miR-202 can induce neuropathic pain through RAP1A [24]. In this study, it has been confirmed that miR-126 functions through targeting EFhd2 according to the prediction of TargetScan and the dual-luciferase assays. Moreover, highly expressed miR-126 significantly suppressed the expression of EFhd2 protein, while decreasing miR-126 expression.

CONCLUSION

MiR-126 expression is downregulated in the sciatic nerve in a mouse model of neuropathic pain. Enhancing miR-126 expression inhibits the inflammatory response and migration of macrophages and alleviated neuropathic pain symptoms in mice. MiR-126 plays its anti-inflammatory and antalgic role mainly by targeting EFhd2 in PSL-induced neuropathic pain.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Ling Fei designed the study and supervised data collection. Wei Huang analyzed and interpreted the data. Ling Fei and Wei Huang prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

Availability of data and materials

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

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