miRNA 146a-5p-loaded poly(D,L-lactic-co-glycolic acid) nanoparticles impair pain behaviors by inhibiting multiple inflammatory pathways in microglia

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Aims: We investigated whether miRNA (miR) 146a-5p-loaded nanoparticles (NPs) can attenuate neuropathic pain behaviors in the rat spinal nerve ligation-induced neuropathic pain model by inhibiting activation of the NF- κ B and p38 MAPK pathways in spinal microglia. **Materials & methods:** After NP preparation, miR NPs were assessed for their physical characteristics and then injected intrathecally into the spinal cords of rat spinal nerve ligation rats to test their analgesic effects. **Results:** miR NPs reduced pain behaviors for 11 days by negatively regulating the inflammatory response in spinal microglia. **Conclusion:** The anti-inflammatory effects of miR 146a-5p along with nanoparticle-based materials make miR NPs promising tools for treating neuropathic pain.

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Neuropathic pain is a debilitating type of chronic pain that often results from chronic neuroinflammation after nerve injury [1]. The pathogenesis of neuropathic pain has been studied extensively and the knowledge on pain has increased rapidly. Early studies focused on neurons and their activity to uncover the mechanisms of neuropathic pain [2]. However, recent studies have identified key roles of microglia in the pathophysiology of neuropathic pain [3,4]. As macrophage-like cells in the CNS, microglia sense the cellular environment and regulate homeostasis in the brain and spinal cord, promoting tissue repair [5]. However, overactivation of microglia under neuroinflammatory conditions can have a detrimental role because of their contribution to the induction and development of neuroinflammation and pain [6,7]. In this case, microglia usually show the M1 (classically activated) phenotype during the early phase and promote inflammation by releasing microglial mediators in response to neuronal activity after nerve injury [8]. These mediators, such as IL-6, IL-1 β and TNF- α , result in cell death, apoptosis and tissue damage [9]. Eventually this activity promotes neuroinflammation and pain behaviors. Inhibiting microglial activation or reducing microglial activity can prevent the development of ongoing pain [10,11].

Recently, miRNA (miR) have emerged as master post-transcriptional regulators [12–14]. miR 146a-5p regulates inflammatory signaling as it acts in post-transcriptional feedback loops that involve many inflammatory genes [15,16]. Two of the best known target genes of miR 146a-5p identified to date are TRAF6 and IRAK1 [17–19]. miR binds to the 3' untranslated regions of these target mRNAs and inhibits their translation or stability, thus downregulating the expression of these proteins. By targeting the adapter proteins TRAF6 and IRAK1, miR 146a-5p can prevent activation of the NF- κ B and p38 MAPK pathways, the two most important inflammatory pathways in activated macrophages [17–19]. The anti-inflammatory role of miR146a-5p has been demonstrated in many previous studies [16,20,21]. Microglia play crucial roles under neuroinflammatory conditions, as activation of the NF- κ B and p38 MAPK pathways in microglia lead to increased release of proinflammatory cytokines, promoting the development of inflammation [6,22]. A 2018 study reported that miR 146a-5p alleviated neuropathic pain behaviors in a chronic constriction injury model [23]. However, it did not report the effects of miR 146a-5p on microglial activity. The function of miR 146a-5p in microglia is still poorly understood.

Our previous studies have revealed that poly(D,L-lactic-co-glycolic acid) (PLGA)-nanoparticles (NPs) are promising tools for delivering siRNA and plasmids into the spinal cord to treat spinal nerve ligation (SNL)-induced neuropathic pain in rats [24,25]. We used the PLGA copolymer as a gene transmission material because of its biodegradability and biocompatibility in humans [13,26] as well as its approval by the US FDA. This method enhances microglia targeting and prolonged pain relief. In this study, we examined whether miR 146a-5p-encapsulated PLGA NPs have therapeutic effects on reducing pain in a similar manner. We expected that miR NPs would mainly target spinal microglia and suppress the activation of multiple inflammatory pathways in these glial cells. Thus, they would have excellent analgesic effects due to multiple mechanisms of action of miR to control inflammatory response in combination with the advantages of PLGA NPs as vehicles for drug delivery.

Materials & methods

Materials

All chemical reagents used in this study were purchased from Sigma-Aldrich (MO, USA) and Thermo Fisher Scientific (MA, USA) unless otherwise noted. Negative control (NC) miRNA (cat. 4464058) and miRNA 146a-5p mimic (cat. 4464067 and assay ID MC10722) for the preparation of PLGA NPs were obtained from Ambion (TX, USA).

miRNA 146a-5p transfection into BV2 cells

The murine microglia cell line BV2 was maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotics. All cultures were kept at 37°C in a humidified incubator under an atmosphere of 5% CO₂. NC miRNA or miRNA 146a-5p mimic was introduced into the BV2 cells with Lipofectamine™ RNAiMAX Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions to reach a concentration of 10 nM. The transfected cells were used for quantitative real-time PCR (qPCR) or western blotting analyses 48 h after infection. Three hours prior to harvesting, the transfected cells in the NC + lipopolysaccharide (LPS) and miRNA + LPS groups were stimulated with LPS.

Western blotting

NC miRNA-/miRNA-146a-5p-treated cells or spinal dorsal horn tissue (L4–L5 segment, 1 cm) were harvested with protein extraction buffer (PRO-PREP; Intron, Daejeon, Korea) 48 h after infection and used for western blotting with anti-TRAF6, anti-IRAK1, anti-phosphorylated-p65 (p-p65), anti-phosphorylated-p38 (p-p38), anti-I κ B- α and anti- β -actin antibodies. Detailed methods are presented in the Supplementary data.

Quantitative real-time PCR

Total RNA was isolated from the ipsilateral dorsal horn of the spinal cord (L4–L5 segment, 1 cm) or BV2 cells with a Hybrid-R kit (GeneAll, Seoul, Korea) and used for cDNA synthesis using TOPscript RT DryMix (Enzynomics, Daejeon, Korea). qPCR was performed and analyzed as described previously [27]. Detailed methods, including primer sequences, are presented in the Supplementary data.

Preparation of PLGA NPs

To prepare PLGA NPs, we added 200 μ l (20 μ M) miRNA 146a-5p mimic or NC miRNA in TE7.5 buffer (Sigma-Aldrich), drop by drop, to 800 μ l dichloromethane (DCM) containing 25 mg PLGA (Corbion, Amsterdam,

The Netherlands) and emulsified it by sonication (UP100H ultrasonic processor; Hielscher Ultrasonics, Teltow, Germany) into a primary W1/O emulsion. Next, 2 ml 2% PVA1500 (w/v) was added directly to the primary emulsion and further emulsified by sonication to form a W1/O/W2 double emulsion. The resulting product was diluted with 6 ml 2% PVA1500 and stirred magnetically for 3 h at room temperature to evaporate the DCM. The resulting PLGA NPs were collected by ultracentrifugation (Optima Max Ultracentrifuge; Beckman Coulter, CA, USA) at $38,000\times g$ for 10 min at 4°C , washed twice with deionized RNase-free water, resuspended in water and finally freeze-dried. To trace the PLGA NPs in cells and spinal cords, we included 1 mg/ml Coumarin 6 in the DCM/PLGA solution. The physical characteristics were analyzed with a ZetaSizer Nano ZS (Malvern Instruments, Malvern, UK) and electron microscopy. The sustained release kinetics of the PLGA NPs and the entrapment efficiency were evaluated as described previously [28].

Animals

Sprague–Dawley rats (6 weeks old, males, 150–200 g) were purchased from Daehan Bio Link (Chung-buk, Korea) and allowed to acclimate to their new environment for 1 week prior to the experiments. All animals were housed (three rats per cage) in a controlled environment with a 12 h light/dark cycle and free access to food and water. The experiments were approved by the Animal Care and Use Committee at Chungnam National University (CNUH-A017-0017) and prepared in accordance with the ethical guidelines of the National Institutes of Health and the International Association for the Study of Pain [29].

Induction of neuropathic pain by L5 spinal nerve ligation

The neuropathic pain model induced by L5 SNL in rats is a well-established model of neuropathic pain [30,31]. Briefly, surgery was performed with isoflurane anesthesia (2% in oxygen mixture; Hana Pharm, Kyung-gi, Korea). The anesthetized rat was placed on its dorsal surface under a microscope. An incision from the L4 to S2 level was made on the back on the left side. The subcutaneous tissue was removed and the paraspinal muscles were exposed toward the left side and then separated with a skin incision 2 cm long. After removal of the L6 transverse process to reveal the L4 and L5 vertebrae, the vertebrae were carefully isolated from each other without damage and the L5 spinal nerve was ligated tightly three-times with 3-0 silk suture (Ethicon, Diegem, Belgium). Complete hemostasis was confirmed, and the wound was sutured. In the sham group, sham surgeries were performed using same technique except the nerves were not ligated.

Pain behavior test with von Frey filaments

The pain behavior test was performed as described previously using von Frey filaments (NC12775-99, Touch Test[®] Sensory Evaluators, 20-Piece Hand Kit; North Coast Medical & Rehabilitation Products, CA, USA). Detailed methods are presented in the Supplementary data.

Intrathecal injection

Intrathecal injection into the lumbar intervertebral space of the spinal cord was performed as described previously [32]. Briefly, rats were placed in the prone position on an operating table under brief isoflurane anesthesia. Next, 20 μl miRNA 146a-5p or NC miRNA-encapsulated PLGA NP solution with different doses of miRNA 146a-5p (0.035, 0.07, 0.1, 0.17 μM) was transferred intrathecally between the fifth and sixth lumbar (L5–L6) intervertebral spaces with a syringe (50 μl ; Hamilton, NV, USA) mounted with a 26 G needle. An obvious tail flick was observed when it was injected successfully. Detailed methods are presented in the Supplementary data.

Immunohistochemistry

Animals were anesthetized with sodium pentobarbital (100 mg/kg, ip.) and perfused with phosphate-buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde (Merck, Darmstadt, Germany) in PBS with a peristaltic pump at a rate of 20 ml/min [33]. The lumbar region (L4–L6) of the spinal cord was obtained and used for immunohistochemical analyses. Detailed methods, including antibody information, are presented in the Supplementary data.

Statistical analyses

Data are expressed as means $\pm$ standard errors of the mean. The statistical significance of differences between two groups was examined by Student's t-test. The statistical significance of differences between multiple groups was

examined by one-way analysis of variance followed by Tukey's *post hoc* test. In all analyses, $p < 0.05$ was taken to indicate statistical significance. All statistical analyses were performed with GraphPad Prism 5 (GraphPad Software, CA, USA).

Results

miRNA 146a-5p imposes negative feedback regulation on LPS-induced upregulation of multiple inflammatory signaling in cultured BV2 cells

To examine the effects of miR 146a-5p on microglia *in vitro*, we cultured BV2 cells and then transfected them with miR mimic or NC miR prior to LPS stimulation. To confirm the efficiency of transfection, we determined miR 146a-5p expression in each group of cultured cells (Figure 1A). In two groups in which cells were transfected with miR mimic (miR and miR+LPS groups), miR 146a-5p expression was significantly increased compared with the NC-miR transfected group. It is important to note that the data shown in Figure 1A also indicate higher miR 146a-5p expression in the NC+LPS group than the NC group following LPS stimulation.

We further investigated the effects of miR 146a-5p on LPS-induced inflammation in BV2 cells by assessing the expression of miR 146a-5p targets (Figure 1B & C). The results of western blotting analyses indicated that expression of both TRAF6 and IRAK1 was upregulated by LPS in the NC+LPS group compared with the NC group (Figure 1B). Meanwhile, transfection of miR mimic effectively blocked LPS-induced upregulation of these proteins in the miR+LPS group. As a result, both the NF- κ B and p38 MAPK pathways were strongly activated in the NC+LPS group in response to LPS treatment, as indicated by higher levels of p-p65, p-p38, active NF- κ B and p38 MAPK molecules (Figure 1B). However, in the miR+LPS group, miR-induced reductions in TRAF6 and IRAK1 expression resulted in inhibition of p-p65 and p-p38 activation. Increased levels of the active molecule p-p65 coincided with the reduction in I κ B- α , one of the two inhibitors of the NF- κ B pathway [34,35]. The depletion of I κ B- α in the NC-LPS group was consistent with the upregulation of p-p65 in this group, whereas miR 146a-5p partially rescued NF- κ B activation-induced I κ B- α depletion in the LPS+miR group (Figure 1B). Quantitative analyses of band intensity shown in Figure 1C also confirmed that LPS induced upregulation of multiple molecules of proinflammatory signaling, including TRAF6, IRAK1, p-p65 and p-p38, in the NC+LPS group. However, overexpression of miR 146a-5p effectively suppressed the protein expression of these molecules in the miR+LPS group.

Activation of the NF- κ B and MAPK pathways in microglia leads to increased release of proinflammatory cytokines, such as IL-6, IL-1 β and TNF- α [36]. As shown in Figure 1D, we further measured and compared the gene expression of microglial mediators by qPCR. Levels of IL-6, IL-1 β and TNF- α released by LPS-activated BV2 cells were strongly increased in the NC+LPS group compared with the NC group. By contrast, miR 146a-5p prevented the increased release of these cytokines in the miR+LPS group. Although a proinflammatory marker and not a cytokine, the regulation of iNOS expression was consistent with that of the proinflammatory cytokines.

Characterization of miRNA 146a-5p/NC miRNA-encapsulated PLGA NPs

To investigate the effects of miR 146a-5p *in vivo*, we used PLGA nanomaterials to introduce miR 146a-5p into SNL rats. miRNA- or NC miRNA-loaded PLGA NPs were prepared by sonication with the typical double emulsion (W/O/W) method, resulting in efficient encapsulation of hydrophilic miRNA/NC miR in PLGA nanoparticles [37]. When measured with a ZetaSizer, the average size and ζ potential of miRNA/NC miR-encapsulated PLGA NPs were 224.1 ± 94.01 nm and -38.5 ± 8.16 mV for miR NPs and 225.3 ± 89.46 nm and -37.1 ± 5.58 mV for NC-NPs, respectively (Figure 2A).

Next, the release profiles of miR NPs were analyzed by using a sustained release kinetics approach. The results in Figure 2B show that miR NPs gradually released miRNA. An initial burst of release (41.8%) was recorded at 24 h, the release of miRNA was consequently observed in a sustained manner at different time points. This cumulative release peaked at 72 h after injection (99.2%). In addition, the percentage of encapsulation efficiency was calculated as the amount of miRNA released from the lyophilized PLGA NPs/the amount of miRNA initially taken to prepare the NPs $\times 100\%$. Encapsulation efficiency was $29.6 \pm 0.24\%$. Furthermore, the integrity and morphology of both miR NPs and NC-NPs were confirmed by scanning electron microscopy (Figure 2C).

Figure 1. miRNA 146a-5p imposes negative feedback regulation on lipopolysaccharide-induced upregulation of multiple inflammatory signaling in cultured BV2 cells. (A) miRNA mimic or NC-miRNA was transfected into BV2 cells with RNAiMAX reagent for 48 h, and miR 146a-5p expression was assessed by qPCR. Data are expressed as mean $\pm$ SEM (one-way ANOVA followed by Tukey's *post hoc* test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs sham). (B) miRNA mimic or NC-miRNA was transfected into BV2 cells with RNAiMAX reagent for 48 h. 3 h prior to harvesting for further investigation, the cells in the LPS treatment groups were stimulated by LPS. Protein expression of miR 146a-5p downstream molecules, including TRAF6, IRAK1, p-p65, p-p38, and IκB-α, was measured with western blotting. (C) Relative band intensities of each protein on the blot in Figure 1B were quantified. (D) Gene expression of microglial mediators, including IL-6, IL-1β, TNF-α, and iNOS, released from LPS-stimulated BV2 cells was determined with qPCR. Data are expressed as mean $\pm$ SEM (one-way ANOVA followed by Tukey's *post hoc* test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs NC + LPS). iNOS: Inducible nitric oxide synthase; LPS: Lipopolysaccharide; NC: Negative control; P-p38: Phosphorylated-p38; P-p65: Phosphorylated-p65; qPCR: Quantitative real-time PCR.

Intrathecal injection of miR 146a-5p-encapsulated PLGA miR NPs into the spinal cord alleviates mechanical allodynia in the SNL-induced neuropathic pain model in a dose-dependent manner

Based on the results obtained *in vitro*, to test whether miR 146a-5p can alter pain behaviors by regulating microglial activity in the spinal cord, we first established a neuropathic pain model in rats by L5 SNL. Following the surgical procedure, von Frey filament tests were conducted on the ipsilateral side of the SNL rats to determine pain

Figure 2. Characterization of miRNA 146a-5p/negative control miRNA-encapsulated poly (D,L-lactic-co-glycolic acid) nanoparticles. **(A)** miRNA or NC miRNA-encapsulated PLGA NPs were dissolved in water and measured for size and ζ potential with a ZetaSizer. **(B)** The release profiles of miR NPs were carried out using a cumulative percentage approach. **(C)** The images show suspended NPs assessed by scanning electron microscopy. Scale bar = 200 nm.
miR NP: miRNA nanoparticle; NC: Negative control; PLGA: Poly(D,L-lactic-co-glycolic acid).

hypersensitivity. As shown in Figure 3A, the mechanical thresholds showed an early decrease on postoperative day (POD) 3 in the SNL rats compared with the sham controls, in which they were maintained up to POD 14. This indicates that the SNL procedure was performed successfully and induced mechanical allodynia in the SNL rats (Figure 3A).

Next we attempted to administer miR to the SNL rats using PLGA NPs. miR NPs were injected intrathecally into the spinal cords of the rats on POD 3, and the mechanical thresholds were determined at different time points after injection. As this was the first time miR NPs had been used in rats with neuropathic pain, it was necessary to determine the effective dose. Different doses of miR-NPs (0.035, 0.07, 0.1 and 0.17 μ M) were administered to the SNL rats. NC miR-encapsulated NPs (NC-NPs) were administered at 0.1 μ M to the rats in the NC-NPs group. Figure 3B & C shows the analgesic effects of the treatments in the six groups: SNL miR NPs (0.035, 0.07, 0.1 and 0.17 μ M), SNL NC-NPs and SNL-untreated groups. A reduction in pain was observed in the three groups

Figure 3. Intrathecal injection of miR 146a-5p-encapsulated poly (D,L-lactic-co-glycolic acid) miRNA nanoparticles into the spinal cord alleviates mechanical allodynia following spinal nerve ligation-induced neuropathic pain in a dose-dependent manner. **(A)** Prior to SNL surgery, rats were subjected to the von Frey filament test and only those that passed a predefined baseline (>10 g) were selected for L5 SNL surgery. Following surgery, the von Frey test was repeated on days 3, 5, 7, 9, 12 and 14 after injury. Data are presented as mean $\pm$ SEM (Student's *t*-test, $***p < 0.001$ vs sham). **(B)** miR NPs with different doses of NC-NPs were injected intrathecally into the spinal cords of the SNL rats on POD 3, and then the von Frey test was conducted in each group of rats on POD 5, 7, 9, 12 and 14. Data are presented as mean $\pm$ SEM (one-way ANOVA followed by Tukey's *post hoc* test, $*p < 0.05$ vs SNL-untreated). **(C)** The mechanical thresholds obtained from each group on POD 9 were compared. Data are presented as mean $\pm$ SEM (one-way ANOVA followed by Tukey's *post hoc* test, $**p < 0.01$, ns, vs SNL-untreated or SNL miR 0.17 μ M). miR NP: MiRNA nanoparticle; NC-NP: Negative control nanoparticle; ns: Not significant; POD: Postoperative day; SNL: Spinal nerve ligation.

treated with 0.07, 0.1 and 0.17 μ M miR NPs. In most groups, the analgesic effects peaked on POD 9 (6 days after injection) and disappeared on POD 14 (11 days after injection). These observations indicate that treatment with miR NPs at these doses effectively improved pain hypersensitivity in the SNL rats. The greatest reduction in pain was achieved with a dose of 0.1 μ M miR NPs on POD 9. The higher dose of 0.17 μ M miR NPs did not have better effects in terms of reducing pain. The lowest dose of 0.035 μ M did not have any analgesic effects and there were no significant differences in pain behavior between the SNL NC-NPs and the SNL-untreated rats.

miR 146a-5p-encapsulated NPs reduce microgliosis & block activation of the NF- κ B & p38 MAPK pathways in spinal microglia after nerve injury

We further investigated the mechanism by which miR reduces pain in SNL rats. We first examined the expression of miR 146a-5p in the spinal dorsal horns of the rats at different time points: POD 3 (before injection), POD 9 (6 days

after injection) and POD 14 (11 days after injection; Figure 4A–C). On POD 3 (before injection), miR 146a-5p was upregulated in the spinal dorsal horns of the SNL rats compared with the sham controls, which suggests an endogenous mechanism of a negative feedback loop of miR 146a-5p on nerve injury (Figure 4A). However, on POD 9 and POD 14, there were no significant differences in miR 146a-5p expression between the SNL NC-NPs and the sham groups. These observations indicate that the SNL procedure induced miR 146a-5p expression in the early phase (POD 3), but these effects were not maintained during the maintenance phase (Figure 4B & C). By contrast, in the SNL rats exposed to miR NPs, miR expression was markedly increased compared with the sham controls and the SNL NC-NPs on POD 9 when the analgesic effects peaked (Figure 4B). However, on POD 14, miR was not significantly increased in the miR NPs compared with other groups (Figure 4C). This correlated with the results of Von Frey filament tests indicating that the analgesic effects of miR NPs treatment peaked on POD 9 and declined on POD 14.

Following confirmation of miR 146a-5p expression, we explored the effects of miR overexpression on spinal microglia on POD 9. After intrathecal injection, miR NPs were expected to mainly influence microglia in the spinal cord and achieve anti-inflammatory effects, as shown in our *in vitro* experiment. To examine this, we first compared microglial activation in the spinal dorsal horns of the sham, NC-NPs and miR NPs groups on POD 9 (Figure 4D). Spinal sections of rats from these three groups were immunostained with anti-iba1 antibody (microglial marker). Figure 4D shows representative images and corresponding iba1 immunostaining levels. On POD 9, microglia were strongly activated in the spinal dorsal horns of the SNL NC-NPs rats compared with the sham controls. Meanwhile, miR NPs reduced the activation of microglia in the SNL miR NPs rats, as indicated by the lower iba1 immunoreactivity in this group (Figure 4D).

To further determine the specific effects of miR on microglial activity, we examined activation of the NF- κ B and p38 MAPK pathways in spinal microglia. Spinal sections were co-stained with anti-iba1 + anti-p-p65 or anti-iba1 + anti-p-p38 antibodies (Figure 4E & F). The immunostaining images in Figure 4E & F show that p-p65 and p-p38 were robustly activated in the spinal dorsal horns of the SNL NC-NPs rats, whereas the sham controls did not show any positive signals for p-p65 or p-p38. In the miR NPs group, p-p65 and p-p38 activation was blocked by miR NPs, as indicated in the staining images as well as the graphs of total p-p65 or p-p38 staining densities. It is important to note that most p-p65 and p-p38 signals overlapped with microglia. This was also confirmed by graphs showing total p-p65/p-p38 + iba1-co-expressing cells/total iba1-expressing cells. These data indicate that the NF- κ B and p38 MAPK pathways were strongly activated in microglia following the SNL procedure. However, treating miR NPs effectively blocked the activation of these pathways, in particular in spinal microglia.

The data in Figure 4 show that miR NPs mainly affected spinal microglia by reducing microglial activation and blocking activation of the NF- κ B and p38 MAPK inflammatory pathways in these glial cells.

Decreased expression of miR 146a-5p downstream molecules & release of microglial mediators in spinal dorsal horns following treatment with miR NPs

To further investigate the mechanism underlying the improvement in pain behaviors in the SNL miR NPs rats by miR, we examined the protein expression of miR 146a-5p targets, including TRAF6 and IRAK1 (Figure 5A). As expected, expression of these adapter proteins increased after the SNL procedure in the SNL NC-NPs rats, but the increases were prevented by treatment with miR NPs in the SNL miR NPs rats. Accordingly, expression of p-p65 and p-p38, which are downstream of TRAF6 and IRAK1, increased in the spinal dorsal horns of the SNL NC-NPs rats, but the effect was reduced by miR NPs. Figure 5A shows the quantification of band intensity for each protein.

The findings shown in Figure 4 indicate that miR NPs mainly targeted microgliosis and microglial activity. As microglia contribute to the development of neuropathic pain by releasing proinflammatory mediators in response to neuronal activity, we examined the release of microglial mediators in the spinal dorsal horns of the rats with qPCR (Figure 5B). Consistent with the results of our *in vitro* experiments, increased release of proinflammatory cytokines, including IL-6, IL-1 β and TNF- α , was detected in the spinal dorsal horns of the SNL NC-NPs rats following nerve injury. By contrast, miR NPs inhibited activation of the NF- κ B and p38 MAPK pathways in microglia and reduced the release of these cytokines. The gene expression of IL-6 and IL-1 β in the SNL miR NPs was significantly lower than in the SNL NC-NPs. Regulation of TNF- α was not significant but was at least consistent with the other cytokines. We also assessed the expression of iNOS, IL-4 and IL-10. iNOS is a proinflammatory marker and is not specifically expressed in microglia [38]. However, in both *in vivo* and *in vitro* experiments, we observed lower levels of iNOS in the miR-treated groups. IL-4 and IL-10 are two antiinflammatory cytokines. Levels of these M2 markers were also increased in the spinal dorsal horns of the SNL NC-NPs rats on POD 9. However, there were no

Figure 4. miRNA nanoparticles reduce microgliosis and block activation of the NF- κ B and phosphorylated-p38 MAPK pathways in spinal microglia after nerve injury. (A) Expression of miR 146a-5p in the spinal dorsal horns of sham and SNL rats was assessed on POD 3. Data are presented as mean $\pm$ SEM (Student's t-test, ** $p < 0.01$ vs sham). (B) On POD 9 (6 days after the injection of NPs), expression of miR 146a-5p was assessed and compared between sham, SNL NC-NPs and the SNL miR NPs groups. Data are presented as mean $\pm$ SEM (one-way ANOVA followed by Tukey's *post hoc* test, * $p < 0.05$, ns vs sham). (C) On POD 14 (11 days after the injection of NPs), expression of miR 146a-5p was assessed and compared between sham, SNL NC-NPs, and the SNL miR NPs groups. Data are presented as mean $\pm$ SEM (one-way ANOVA followed by Tukey's *post hoc* test, ns vs sham). (D) On POD 9, microglial activation in the spinal cords of the rats in the sham, SNL NC-NPs, and SNL miR NPs groups was assessed by immunostaining and confirmed by iba1 band density in the graph. Scale bars = 100 μ m (left) and 50 μ m (right). Data are presented as mean $\pm$ SEM (one-way ANOVA followed by Tukey's *post hoc* test, *** $p < 0.001$ vs SNL NC-NPs). (E) Spinal cord sections of sham, SNL NC-NPs and SNL miR NPs groups were co-stained with anti-p-p65 and anti-iba1 antibodies. Scale bars = 50 μ m (left) and 20 μ m (right). The band densities of total p-p65 and p-p65+iba1+/iba1+ were quantified. Data are presented as mean $\pm$ SEM (one-way ANOVA followed by Tukey's *post hoc* test, *** $p < 0.001$ vs SNL NC-NPs). (F) Spinal cord sections of sham, SNL NC-NPs and SNL miR NPs groups were co-stained with anti-p-p38 and anti-iba1 antibodies. Scale bars = 50 μ m (left) and 20 μ m (right). The band densities of total p-p38 and p-p38+iba1+/iba1+ were quantified. Data are presented as mean $\pm$ SEM (one-way ANOVA followed by Tukey's *post hoc* test, *** $p < 0.001$ vs SNL NC-NPs). miR NP: miRNA nanoparticle; NC-NP: Negative control nanoparticle; ns: Not significant; POD: Postoperative day; P-p38: Phosphorylated-p38; P-p65: Phosphorylated-p65; SNL: Spinal nerve ligation.

Figure 5. Decreased expression of miRNA 146a-5p downstream molecules and release of microglial mediators in spinal dorsal horns following treatment of miRNA nanoparticles. (A) Protein expression of miR 146a-5p downstream molecules, including TRAF6, IRAK1, p-p65, p-p38 and IκB-α, in the spinal dorsal horns of sham, SNL NC-NPs and SNL miR NPs groups and the corresponding quantitative band densities were measured on POD 9 by western blotting. Data are presented as mean ± SEM (one-way ANOVA followed by Tukey's *post hoc* test, **p* < 0.05; ***p* < 0.01 vs SNL NC-NPs). **(B)** Gene expression of microglial mediators, including IL-6, IL-1β, TNF-α, iNOS, IL-4 and IL-10, in the spinal dorsal horns of sham, SNL NC-NPs and SNL miR NP groups was assessed with qPCR. Data are presented as mean ± SEM (one-way ANOVA followed by Tukey's *post hoc* test, **p* < 0.05; ***p* < 0.01, ns vs SNL NC-NPs). miR NP: miRNA nanoparticle; NC-NP: Negative control nanoparticle; ns: Not significant; POD: Postoperative day; P-p38: Phosphorylated-p38; P-p65: Phosphorylated-p65; qPCR: Quantitative real-time PCR; SNL: Spinal nerve ligation.

significant differences in the expression of these cytokines between the NC-NPs and miR NPs groups. In our *in vitro* experiment with BV2 cells, we also found no regulation of the anti-inflammatory response in the miR-transfected groups (data not shown). These findings indicate that miR 146a-5p modulates the proinflammatory response only and has no effects on the anti-inflammatory process.

Discussion

Neuropathic pain is a chronic pain that occurs after nerve damage or because of a malfunction in the nervous system. Although the pathological mechanism of neuropathic pain has been studied in detail, treatments for it have yet to be developed. Several types of drugs, including anticonvulsants, antidepressants, nonsteroidal anti-inflammatory drugs and opioids, are used to clinically treat neuropathic pain [39]. These drugs mainly act to modulate synaptic plasticity

or inhibit neuronal excitation [40]. Over the past decade, there has been a rapid increase in microglial studies on neuropathic pain, which suggests a central role of microglia in the pathogenesis of neuropathic pain [7]. Microglia contribute to the development of pain by producing microglial mediators that can rapidly modulate inflammatory responses in the CNS. A number of studies have focused on methods of disturbing microglial activity as a means of improving pain behaviors. miR 146a-5p is a master post-transcriptional regulator of many genes involved in immune and inflammatory responses. It is interesting that before examining the effects of miR 146a-5p on BV2 cells, we checked the effects on LPS-induced inflammation in the RAW 264.7 macrophage cell line. miR 146a-5p prevented upregulation of TRAF6 and IRAK1, which subsequently reduced TNF- α secretion in LPS-stimulated RAW 264.7 cells (Supplementary Figure 1). The results of the present study demonstrate that miR 146a-5p was successfully overexpressed in BV2 cells by transfection of miR mimic and that overexpression of miR repressed LPS-induced inflammatory response in these cells. NF- κ B and p38 MAPK are two downstream signaling pathways that are activated after upregulation of TRAF6 and IRAK1 molecules [19,41]. miR 146a-5p downregulated the expression of target genes, including TRAF6 and IRAK1, subsequently reducing activation of the NF- κ B and p38 MAPK pathways and the release of proinflammatory cytokines. Our *in vitro* data also showed that miR 146a-5p expression in the NC+LPS group was induced in BV2 in response to LPS stimulation, forming a negative feedback loop to control microglial activity. This observation is consistent with previous studies showing that miR 146a-5p was induced by proinflammatory stimulation and that it participated in a negative feedback loop to control the inflammatory response [42–44].

We examined whether miR 146a-5p can drive pain behaviors in a neuropathic pain model in rats by regulating spinal microglial activity in a similar manner. As expected, all data obtained in the *in vivo* experiment on SNL rats supported this. miR effectively reduced microgliosis and microglial activity by targeting multiple inflammatory pathways in these cells. Both NF- κ B and p38 MAPK were suppressed by miR NPs, thereby preventing the release of microglial mediators, which play important roles in the development of neuroinflammation and pain. We also found that miR expression was upregulated in the spinal dorsal horns of the SNL rats on POD 3, which suggests an endogenous mechanism against inflammation. However, these effects only lasted through the early phase and were not maintained during the maintenance phase, in which overexpression of microglia in response to nerve injury results in excessive neuroinflammation. We did not detect significant differences in miR expression between the sham and SNL NC rats on POD 9 and POD 14. However, treatment with miR NPs led to overexpression of miR and therefore inhibited the inflammatory response, in particular in spinal microglia.

It is very important to examine how miR was delivered into the spinal cords of the rats. Our previous studies introduced a novel nanomaterial-based approach to treating neuropathic pain [24,25]. PLGA nanoparticles have been approved by the FDA and are easily endocytosed by microglial cells [26]. Our previous studies, in which p38 siRNA- or Foxp3 plasmid-loaded PLGA NPs were injected intrathecally into the spinal cords of SNL rats, showed that PLGA NPs mainly targeted microglia, not neurons or astrocytes. We have also demonstrated the safety and nontoxicity of our PLGA NPs [24,25]. Taking advantage of genetic interference and nanomaterial vehicle, we administered miR 146a-5p-encapsulated PLGA NPs to SNL rats. The data obtained from the *in vivo* experiments demonstrate that miR NPs were successfully delivered into the spinal microglia and imposed negative feedback regulation on the inflammatory response in these glial cells. It is worth noting that miR NPs reduced pain behaviors in rats in a dose-dependent manner, with a dose of 0.1 μ M miR having the greatest effects on pain reduction on POD 9. Therefore, we administered this dose to the SNL rats in most of the subsequent investigations.

One point for discussion is that miR NPs improved pain behaviors in the von Frey filament test for approximately 11 days after one intrathecal injection on POD 3. In a previous study with a chronic constriction injury-induced neuropathic pain model, the analgesic effects of miR 146a-5p on pain behaviors were observed from POD 7 to POD 21 with four direct intrathecal injections of original miR [23]. This suggests that our miR 146a-5p-loaded NPs reduced pain longer than the original drug. Note that the analgesic effects of most drugs that have been clinically prescribed to treat neuropathic pain often last for only several hours because of their short biological half-life. Our PLGA NP-based treatment had better analgesic effects that lasted for several days. These long-term effects may be due to the slow release of the drug from nanoparticles within the cell along with the therapeutic effects of miRNAs, siRNAs or plasmid vectors. Further investigations are required to determine the molecular mechanism by which PLGA NPs achieve their sustained analgesic effects.

Conclusion

We have shown that PLGA NPs have promise for drug delivery. miR 146a-5p-encapsulated NPs improved pain behaviors by inhibiting multiple inflammatory pathways in spinal microglia, subsequently reducing the release of proinflammatory cytokines. We believe that miR 146a-5p with its anti-inflammatory effects represents a novel therapeutic candidate for treating neuropathic pain.

Summary points

- Neuropathic pain is a chronic pain caused by damage to or disease affecting the somatosensory nervous system. It is usually very difficult to treat and only a small percentage of patients achieve partial relief.
- miRNA 146a-5p is a master posttranscriptional regulator of many genes involved in immune and inflammatory responses. It affects the translation or stability of TRAF6 and IRAK1 adapter proteins, which can activate the NF- κ B and p38 MAPK pathways within cells. This prevents the release of proinflammatory cytokines.
- In previous studies, we found that poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) are promising vehicles for drug delivery. In the present study, we used miR 146a-5p-loaded PLGA NPs to target microglia in the spinal cords of spinal nerve ligation (SNL) rats to reduce neuropathic pain.
- Transfection of miR 146a-5p mimic efficiently reduced lipopolysaccharide-induced upregulation of multiple inflammatory molecules in BV2 cells.
- The physical characteristics of miRNA 146a-5p NPs were analyzed by ζ potential measurement and scanning electron microscopy. miR 146a-5p-encapsulated PLGA NPs injected intrathecally into the spinal cords of SNL rats on day 3 after surgery attenuated pain behaviors in the von Frey filament test for 11 days.
- Microglial activation and activation of the NF- κ B and p38 MAPK pathways in spinal microglia were blocked by miR NPs, which resulted from decreased expression of TRAF6 and IRAK1 adapter proteins, followed by reduced release of microglial mediators.
- miR NPs had good analgesic effects in SNL rats that lasted for several days. This strongly suggests that miRNA 146a-5p with its anti-inflammatory effects may represent a therapeutic option for treating neuropathic pain and that the use of NPs represents a novel approach to drug delivery.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/nnm-2019-0462

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