Overexpression of microRNA-141 relieves chronic constriction injury-induced neuropathic pain via targeting high-mobility group box 1

JIZHENG ZHANG¹, HUA ZHANG¹ and TINGTING ZI^2

¹Department of Anesthesiology, Tianjin Hospital, Tianjin 300211; ²Department of Anesthesiology, Beijing Children's Hospital, Capital Medical University, Beijing 100045, P.R. China

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Abstract. The function of microRNAs (miRNAs or miRs) in regulating neuropathic pain has attracted increasing attention in recent years. However, the precise mechanism of miRNAs in neuropathic pain remains largely unknown. In the present study, an important role of miR-141 and its putative target gene, high-mobility group box-1 (HMGB1), was demonstrated in a rat model of neuropathic pain induced by chronic constriction injury (CCI). The expression of miR-141 was significantly downregulated in the dorsal root ganglion of rats following CCI surgery. Overexpression of miR-141 by intrathecal injection of miR-141 precursor mediated by a lentivirus-derived gene transfer significantly inhibited mechanical allodynia, thermal hyperalgesia and proinflammatory cytokine release in CCI rats. Using a dual luciferase reporter assay, a direct interaction between miR-141 and the 3'-untranslated region of HMGB1 was verified. Overexpression of miR-141 significantly suppressed the expression of HMGB1 in vitro and in vivo. Furthermore, overexpression of HMGB1 apparently abrogated the beneficial effect of *miR-141* on inhibiting neuropathic pain. Taken together, the data suggest that overexpression of miR-141 alleviates neuropathic pain development via targeting and inhibiting HMGB1, implying that blocking HMGB1 by miR-141 could be a useful therapeutic strategy for the treatment of neuropathic pain.

Introduction

Chronic neuropathic pain has previously been characterized by hyperalgesia and allodynia has become a notable public health problem that affects a broader population worldwide (1,2). Generally, chronic neuropathic pain is a consequence of a disease or lesion that causes damage to the somatosensory system (3). The increasing amount of evidence has indicated that neuropathic pain contributes to the pain experience for a subset of the osteoarthritis population (4). In addition, neuropathic pain has been suggested to be an underestimated problem in patients following total knee replacement (5). However, all current therapies for neuropathic pain are far from effective and only treat the symptoms (6). The main obstacle hampering the development of effective therapeutics is that the precise molecular mechanism underlying neuropathic pain remains to be elucidated.

MicroRNAs (miRNAs or miRs) are a subset of small non-coding RNAs with a length of ~21 nucleotides that regulate the expression of numerous genes via targeting the 3'-untranslated region (UTR) of messenger RNA (mRNA), resulting in mRNA destabilization and degradation, and thus protein translational inhibition (7,8). miRNAs have been found to be involved in regulating numerous cellular processes that could participate in the pathogenesis of various diseases (9,10). In recent years, the role of miRNAs in neuropathic pain has been highlighted (11). It has been reported that the expression of >63 miRNAs was significantly altered in a rat model of neuropathic pain (12). miR-182, miR-18 and miR-96 have been revealed to be highly expressed in the dorsal root ganglion (DRG) in a rat model of neuropathic pain (13). Favereaux et al (14) demonstrated that miR-103 was decreased in neuropathic animals and intrathecal injection of miR-103 successfully attenuated neuropathic chronic pain. More recently, Tan et al (15) reported that inhibition of miR-155 relieved neuroinflammation and neuropathic pain development by upregulating suppressor of cytokine signaling 1 expression. All these findings suggest that miRNAs may be served as a potential and effective molecular target for developing novel therapies for treatment of neuropathic pain.

High-mobility group box 1 (HMGB1) has been considered as an abundant and ubiquitous non-histone DNA-binding protein that is expressed in numerous cell types, including neurons and glial cells (16). Emerging evidence has reported that HMGB1 is extensively involved in regulating proinflammatory diseases, such as rheumatoid arthritis (17) and sepsis (18). Therefore, HMGB1 has been suggested as an alarmin to orchestrate inflammatory responses that

Correspondence to: Dr Hua Zhang, Department of Anesthesiology, Tianjin Hospital, 406 Jiefangnan Road, Tianjin 300211, P.R. China E-mail: huazhang_hz@163.com

Abbreviations: HMGB1, high-mobility group box 1; miRNAs, microRNAs; DRG, dorsal root ganglion; UTR, untranslated region; CCI, chronic constriction injury

Key words: microRNA-141, high-mobility group box 1, chronic constriction injury, neuropathic pain

regulate cell migration, phagocytosis and activation of immune cells (19). With consideration of the important role of HMGB1 in inflammation, HMGB1 is expected to be involved in regulating neuropathic pain, which is characterized as an excessive proinflammation in the nervous system (20-22). Initial reports revealed that exogenous HMGB injection induced neuropathic pain-like behavior in rodents (23). Shibasaki *et al* (24) demonstrated that induction of *HMGB1* in DRG contributes to neuropathic pain following peripheral nerve injury. In a rat model of tibial nerve injury, HMGB was redistributed from the nucleus to the cytoplasm in neurons that contributed to tactile hyperalgesia (25). Administration of the HMGB1 neutralization antibody effectively reduced neuroinflammation and improved the pain-related behavior (26). Therefore, HMGB1 may be a promising therapeutic target for neuropathic pain.

The present study identified *HMGB1* as a predicated target gene of *miR-141* by bioinformatics analysis (http://www. targetscan.org), implying that *miR-141* may regulate *HMGB1* expression and thus participate in neuropathic pain. Therefore, the study was designed to identify and validate whether *miR-141* directly regulated *HMGB1* expression and participated in the development of neuropathic pain, and aimed to provide an effective and potential molecular target for the treatment of neuropathic pain.

Materials and methods

Animals. Adult male Sprague-Dawley rats, weighing 220-250 g, were provided by the Laboratory Animal Center of Tianjin Medical University (Tianjin, China). The animals were raised at room temperature of $24.0\pm1^{\circ}$ C with a 12/12-h light/dark-cycle and free access to food and water. All the animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Tianjin Hospital (Tianjin, China).

DRG culture. The primary DRG neurons were isolated and cultured according to a previously reported method (27). Briefly, the bilateral DRG from rats were quickly dissected under the microscope and digested for 15 min at 37°C in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA) containing trypsin (1 mg/ml) and collagenase (2 mg/ml) (Sigma, St. Louis, MO, USA). The DRG neurons were obtained by a pasteur pipette (Shanghai Lianshuo Biological Technology Co., Ltd., Shanghai, China), and were plated in 6-well plates at 1x106 cells/well in DMEM containing 5 μ g/ml cytarabine (Hisunpharm, Taizhou, China) for 24 h to suppress the growth of non-neuronal cells. Subsequently, cells were collected and cultured in DMEM/F-12 (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco), 0.1 mg/ml L-glutamine, and 10 ng/ml nerve growth factor (Life Technologies) supplemented with 1% penicillin/streptomycin (Sigma).

Chronic constriction injury (CCI) model. A rat model of neuropathic pain was established by CCI according to a previously described method (28). Briefly, rats were anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital (40 mg/kg; Merck, Darmstadt, Germany). The left sciatic nerve was exposed and ligated with 4-0 catgut thread (Johnson &

Johnson, New Brunswick, NJ, USA) at 4 sites with an interval of 1 mm. Sham-operated rats were performed with left sciatic nerve exposure, without ligation.

Intrathecal catheter implantation. Intrathecal catheter implantation was performed according to a method reported previously (29). Briefly, rats were anaesthetised with 40 mg/kg sodium pentobarbital (i.p.). The occipital muscles were separated to expose the cisternal membrane. The polyethylene catheter (PE-10; American Health & Medical Supply International Corp., New York, NY, USA) was inserted in the cisterna magna through an incision and advanced 7.0-7.5 cm caudally to the lumbar enlargement. The intrathecal implantation was verified by paralysis of the bilateral hind limbs with injection of 2% lidocaine (Sigma). Subsequently, the catheter was fixed and the incision was sealed. Intrathecal lentiviral (LV)-miR-141 (GenePharma, Shanghai, China) administration was performed using a microinjection syringe linked with the intrathecal catheter. A total of 10 μ l of recombinant lentivirus was administrated once daily for 3 days after CCI. For biological analysis, the rats were euthanized quickly and the L4-L5 lumbar spinal cords were removed and the DRG were dissected.

Examination of pain threshold. Mechanical allodynia indicated by the paw withdrawal threshold was detected according to a method previously described (30). Briefly, rats were plated on a metal mesh floor in a transparent plastic box. The pressure was created using the electronic von Frey filament (IITC, Woodland Hills, CA, USA) to the plantar surface of each hind. The time of paw withdrawal of each rat in response to the force was recorded. The paw withdrawal latency in response to radiant heat was measured according to the Hargreaves method (31). The rats were placed in a perspex box on an elevated glass, and a radiant heat source was focused on mid-plantar area underneath the glass. The duration between the start of stimuli and paw withdrawal was read and recorded by a digital timer. A cut-off time was set at 20 sec of irradiation to avoid tissue damage according to a standard procedure (32).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNAs and miRNAs were extracted using mirVanaTM miRNA isolation kit (Life Technologies) according to the manufacturer's instructions. The cDNA was generated by a TaqMan miRNA reverse transcription kit (Life Technologies) according to the manufacturer's instructions. RT-qPCR was performed using SYBR Premix Ex Taq (Takara, Dalian, China). The relative quantification of gene expression level was compared with the internal reference GAPDH (for mRNA) or U6 snRNA (for miRNAs) using the 2^{- $\Delta\DeltaCt$} method.

Enzyme-linked immunosorbent assay (ELISA). The concentrations of interleukin (IL)-1 β , IL-6 and tumor necrosis factor (TNF)- α in the lumbar spinal cords were measured by corresponding ELISA kits (R&D Systems, Minneapolis, MN, USA) as described by the manufacturer's instructions. The absorbances at 450 nm were read using an ELISA reader (Bio-Tek, Winooski, VT, USA).

Western blot analysis. The protein in each sample was extracted using a protein extraction kit (Applygen Technologies, Beijing,

China). Protein concentration was measured using the Bradford method. For protein separation, a total of 25 μ g of protein was loaded on 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by transfer to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), which was subsequently blocked with 2.5% non-fat milk for 1 h at 37°C. Following this, primary antibodies were added and incubated at 4°C overnight. Horseradish peroxidase-conjugated secondary antibodies (1:2,000; bs-0295G-HRP; Bioss, Beijing, China) were added and the sample was incubated for 1 h at room temperature. Following three washes with Tris-buffered saline Tween-20, the immune-reactive protein bands on the membrane were visualized using an enhanced chemiluminescence detection system (Amersham, Little Chalfont, UK). The primary antibodies used in the experiment were as follows: anti-p65 (sc-372; Santa Cruz Biotechnology, Dallas, TX, USA), anti-p-p65 (#3031; Cell Signaling Technology, Danvers, MA, USA), anti-HMGB1 (ab79823; Abcam, Cambridge, UK) and anti-GAPDH (bs-13282R; Bioss). Relative protein expression was quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Dual-luciferase reporter assay. The cDNA fragments of *HMGB1* 3'-UTR containing the putative binding site of *miR-141* were amplified and subcloned into the pGL3 luciferase promoter vector (Promega, Madison, WI, USA). The pGL3-*HMGB1* was co-transfected with LV-*miR-141* into the human embryonic kidney 293 cells for 48 h. The human embryonic kidney 293 cells for 48 h. The human embryonic kidney HEK293 cells were obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/ streptomycin (Sigma). Subsequently, cells were harvested and lysed in which the luciferase activity was quantified using the dual-luciferase reporter system (Promega) according to the manufacturer's instructions.

Data analysis. Data are expressed as mean \pm standard deviation and processed using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA). Statistical differences between two groups were analyzed by Student's t-test. Statistical differences among multiple groups were analyzed by one-way analysis of variance followed by Bonferroni post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-141 is downregulated in the DRG in CCI rats. To examine whether *miR-141* has a potential role in regulating neuropathic pain, the expression profiles of *miR-141* in DRG in CCI rats were examined using RT-qPCR. The results showed that *miR-141* expression was downregulated in the DRG of CCI rats as compared with that from sham-operated rats at postoperative days 1, 3, 7 and 14 (Fig. 1). The data suggest that *miR-141* may have an important role in regulating neuropathic pain.

Overexpression of miR-141 attenuates mechanical allodynia and thermal hyperalgesia in CCI rats. To investigate whether targeting miR-141 expression has a beneficial effect on neuropathic pain, the CCI rats were subjected to intrathecal injection







Figure 2. Effect of *miR-141* overexpression on neuropathic pain in CCI rats. (A) Reverse transcription-quantitative polymerase chain reaction analysis of *miR-141* expression level in the dorsal root ganglion from CCI rats subjected to intrathecal injection of LV-*miR-141* at days 7 and 14 post-CCI surgery. N=3, *P<0.05 vs. LV-miR-Ctrl group. (B) Mechanical allodynia indicated by PWT in response to von Frey filaments stimulation was examined. PWT was measured at days 0, 1, 3, 7 and 14 post-CCI surgery. The paw withdrawal force (indicated by g) was determined and recorded in each group. N=6, *P<0.05 vs. LV-miR-Ctrl group. (C) Thermal hyperalgesia indicated by PWL in response to radiant heat was determined. The latency of paw withdrawal (indicated by sec) was detected and recorded in each group at each time point. N=6, *P<0.05 vs. LV-miR-Ctrl group. CCI, chronic constriction injury; PWT, paw withdrawal threshold; PWL, paw withdrawal latency.

of lentivirus-mediated transfer of *miR-141* (LV-*miR-141*) or non-specific control miRNAs (LV-miR-Ctrl). The expression level of *miR-141* was significantly increased in the LV-*miR-141* group in comparison with the LV-miR-Ctrl group at postoperative day 7 and 14 (Fig. 2A). The neuropathic pain development indicated by mechanical allodynia (Fig. 2B) and thermal hyperalgesia (Fig. 2C) was markedly inhibited by *miR-141* overexpression. The data suggest that overexpression of *miR-141* is capable of attenuating neuropathic pain.



Figure 3. Effect of *miR-141* on inflammation in CCI rats. Concentrations of (A) TNF- α , (B) IL-1 β and (C) IL-6 in rat spinal cords from different groups were detected by ELISA at day 7 post-CCI surgery. The data are indicated as pg/ml total proteins. (D) Western blot analysis of phosphorylated p65 protein level in the rat spinal cords from different groups. Relative protein expression of p-p65 normalized with (E) GAPDH and (F) total p65 was quantified using Image-Pro Plus 6.0 software. N=3, *P<0.05 vs. sham group; *P<0.05 vs. LV-miR-Ctrl. CCI, chronic constriction injury; TNF, tumor necrosis factor; IL, interleukin; LV, lentivirus.

A



Figure 4. miR-141 directly targets HMGB1. (A) Schematic representation of the putative binding sites between miR-141 and HMGB1 3'-UTR. (B) Dual-luciferase activity reporter assay to verify the interaction between miR-141 and 3'-UTR of HMGB1. The wild-type or mut 3'-UTR of HMGB1in pGL3 luciferase reporters were transfected into HEK293 cells with LV-miR-141 for 48 h. N=3, *P<0.05 vs. LV-miR-Ctrl. HMGB1, high-mobility group box 1; UTR, untranslated region; mut, mutant; LV, lentivirus.

Overexpression of miR-141 inhibits proinflammatory cytokine expression in CCI rats. To further explore the effect of miR-141 overexpression on neuropathic pain development, the expression of proinflammatory cytokines in the spinal cord of CCI rats was analyzed. The results showed that the protein concentrations of TNF- α (Fig. 3A), IL-1 β (Fig. 3B) and IL-6 (Fig. 3C) in rat spinal cord that were significantly elevated in CCI rats were significantly decreased by *miR-141* overexpression, as detected by ELISA. Furthermore, the activity of proinflammatory transcription factor NF- κ B p65 indicated by phosphorylation of p65 was also significantly decreased by *miR-141* overexpression (Fig. 3D-F). These results indicate that overexpression of *miR-141* suppresses neuroinflammation in CCI rats.

miR-141 targets the 3'-UTR of HMGB1 and regulates HMGB1 expression DRG neurons in vitro. To investigate the potential underlying mechanism of miR-141 in regulating neuropathic pain, the putative target gene of miR-141 was screened and HGMB1, which has been suggested to be an important proinflammatory mediator in regulating neuropathic pain (33), contained the predicted targeting sequences in the 3'-UTR (Fig. 4A). To verify that this association was authentic, a dual-luciferase reporter assay was performed. The results demonstrated that overexpression of miR-141 significantly inhibited the luciferase activity in pGL3-HMGB1 3'-UTR transfected cells, whereas it had no apparent effect on pGL3-mut HMGB1 3'-UTR transfected cells (Fig. 4B). Additionally, the regulatory effect of miR-141 on HMGB1 expression in cultured DRG neurons was further detected. RT-qPCR analysis showed that the mRNA expression of HMGB1 was significantly inhibited in LV-miR-141 infected cells (Fig. 5A), as compared with LV-miR-Ctrl infected cells. The effect of miR-141 overexpression on HMGB1 protein



Figure 5. Effect of *miR-141* overexpression on HMGB1 expression in DRG neurons *in vitro*. Detection of the (A) mRNA and (B) protein expression level of HMGB1 in DRG neurons infected with LV-*miR-141* for 48 h. LV-miR-Ctrl was used as the control. Relative protein expression was quantified using Image-Pro Plus 6.0 software. N=3, *P<0.05 vs. LV-miR-Ctrl. HMGB1, high-mobility group box 1; DRG, dorsal root ganglion; LV, lentivirus.



Figure 6. *miR-141* regulates *HMGB1* expression in CCI rats *in vivo*. The expression of HMGB1 (A) mRNA and (B) protein levels in the DRG from CCI rats subjected to intrathecal injection of LV-*miR-141* at day 7. N=3, *P<0.05 vs. LV-miR-Ctrl. (C) Western blot analysis of HMGB1 protein expression levels in the DRG from LV-*miR-141* and LV-HMGB1 co-infected CCI rats. Relative protein expression was quantified using Image-Pro Plus 6.0 software. LV-Ctrl encoding non-specific protein was used as control. N=3, *P<0.05 vs. LV-*miR-141*+LV-Ctrl. HMGB1, high-mobility group box 1; CCI, chronic constriction injury; DRG, dorsal root ganglion; LV, lentivirus.



Figure 7. Overexpression of HMGB1 restores neuropathic pain reduced by *miR-141*. (A) PWT and (B) PWL was detected at days 0, 1, 3, 7 and 14 post-CCI in CCI rats infected with LV-*miR-141* with or without LV-HMGB1. N=6, *P<0.01 vs. LV-*miR-141*+LV-Ctrl. HMGB1, high-mobility group box 1; PWT, paw withdrawal threshold; PWL, paw withdrawal latency; CCI, chronic constriction injury; LV, lentivirus.

expression was also validated by western blot analysis, which demonstrated that the HMGB1 protein expression level was markedly decreased by *miR-141* overexpression (Fig. 5B).

Overexpression of miR-141 regulates HMGB1 expression in the DGR of neuropathic pain in rats in vivo. To ascertain the possible role of miR-141 in regulating HMGB1 expression, the effect of miR-141 overexpression was further analyzed on HMGB1 expression in CCI rats in vivo. The results showed that intrathecal injection of LV-miR-141 significantly decreased the mRNA (Fig. 6A) and protein (Fig. 6B) expression of HMGB1 in the DRG from CCI rats. To further clarify that miR-141 had an important role in regulating neuropathic pain via regulating HMGB1, a rescue experiment was performed by co-infection of the rats with LV-miR-141 and LV-HMGB1 containing no specific targeting sites of miR-141 in 3'-UTR. The results showed that HMGB1 protein expression was overexpressed in LV-HMGB1-infected rats (Fig. 6C). Additionally, HMGB1 overexpression restored the neuropathic pain development reduced by miR-141 overexpression (Fig. 7A and B).

Discussion

The present study demonstrated that overexpression of *miR-141* significantly attenuated neuropathic pain via targeting and inhibiting the expression of *HMGB1*. The downregulated expression of *miR-141* was responsible for the highly increased expression of *HMGB1*, whereas overexpression of *miR-141* significantly inhibited the upregulated expression of *HMGB1* and suppressed the mechanical allodynia and thermal hyperalgesia, as well as the proinflammatory cytokines release in neuropathic pain rats. In addition, overexpression of *HMGB1* abolished the protective effect of *miR-141* overexpression on

neuropathic pain that further confirmed the direct interaction between *miR-141* and *HMGB1* in neuropathic pain. The present data implied that blocking *HMGB1* by *miR-141* could be a useful therapeutic strategy for neuropathic pain.

Previously, the role of miRNAs in regulating neuropathic pain has been widely studied (34,35). Im et al (36,37) reported that ectopic miR-23b expression ameliorates neuropathic pain by inhibiting nicotinamide adenine dinucleotide phosphate oxidase 4 in the spinal cord. Intrathecal miR-124 treatment prevented persistent inflammatory and persistent hyperalgesia in chronic hyperalgesia mice (38). Upregulated miR-195 was identified in the spinal microglia of rats with spinal nerve ligation and the miR-195 inhibitor prevented neuronal inflammation and neuropathic pain through increasing autophagy (39). Intrathecal injection of miR-96 has been reported to inhibit neuropathic pain of CCI rats via inhibiting Nav1.3 expression (27). More recently, miR-155 was found to be highly expressed in the spinal cord of CCI rats and administration of miR-155 inhibitor attenuated neuropathic pain and proinflammatory cytokine expression through regulating the suppressor of cytokine signalling 1, which was an inhibitor of proinflammation (15). In the present study, miR-141 expression was altered in CCI rats and intrathecal administration of lentivirus expressing miR-141 reversed the neuropathic pain and neuronal inflammation in CCI rats. The role of miR-141 in diseases has been widely studied, such as cancer (40,41). Certain studies also indicated an important role of miR-141 in regulating inflammation-associated diseases (42-44). The present study revealed that HMGB1 was a direct target gene of miR-141, both of which were involved in neuropathic pain.

As the critical role of HMGB1 has been reported in neuropathic pain (23), numerous strategies targeting HMGB1 have been carried out to treat neuropathic pain. A neutralizing antibody against HMGB1 (anti-HMGB1) has been shown to successfully alleviate the mechanical allodynia in rats with spinal nerve ligation (24). Treatment of anti-HMGB1 neutralization antibody significantly inhibited proinflammatory cytokine expression in the DRG and improved the pain-related behavior (26). Ren et al (45) reported that intrathecal injection of anti-HMGB1 repressed mechanical allodynia induced by diabetes. Nakamura et al (46) provided evidence that intravenous injection of anti-HMGB1 relieved neuropathic pain in rats followed by partial sciatic nerve ligation. Furthermore, anti-HMGB1 also showed an effective anti-allodynia effect in a rat model of bone cancer pain (47). In addition, it has been reported that Tanshinone IIA reversed thermal hyperalgesia and mechanical allodynia induced by spinal nerve ligation via modulating HMGB1 and its receptor, Toll-like receptor 4 (48). Intrathecal injection of lentivirus-mediated transfer of IL-10 inhibited neuropathic pain via regulating spinal HMGB1 expression in CCI rats (49). All the aforementioned studies indicate that HMGB1-based therapeutic strategies could be an effective method for treatment of neuropathic pain. In the present study, miR-141 could regulate HMGB1 expression through directly targeting the 3'-UTR of HMGB1. In addition, intrathecal injection of lentivirus-mediated transfer of miR-141 significantly attenuated neuropathic pain and proinflammatory cytokine release, including TNF- α , IL-1 β and IL-6, in the spinal cord of CCI rats.

Inhibiting HMGB1 by miRNAs has been reported in various studies. For instance, *miR-22* repressed osteosarcoma via targeting HMGB1 (50). *miR-181b* has been reported to regulate drug sensitivity of acute myeloid leukemia by targeting and inhibiting HMGB1 (51). However, the present data suggested that *miR-141* could target and inhibit HMGB1 expression in DRG neurons *in vitro* and *in vivo*. In conclusion, the present data revealed that *miR-141* was significantly decreased in the DRG of CCI rats, which directly regulated HMGB1 expression and implied that targeting *miR-141*-HGMB1 could be a useful therapeutic strategy for the treatment of neuropathic pain.

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