MicroRNA-143-3p contributes to the regulation of pain responses in collagen-induced arthritis

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Abstract. Patients with rheumatoid arthritis (RA) suffer from pain, which is associated with inflammation, peripheral and central pain processing, and joint structure damage. The aim of the present study was to investigate a key microRNA (miR) and its target genes that are involved in the pain responses of RA, and to clarify the mechanism of pain regulation. Collagen-induced arthritis (CIA) was induced in DBA/1 and C57BL/6 mice. The paw swelling, mechanical withdrawal threshold (MWT), thermal withdrawal latency (TWL), and expression levels of tumor necrosis factor (TNF)-α and prostaglandin (PG)E_2 in the sera were investigated. Decreased MWT and TWL, and increased TNF-α and PGE_2, in the CIA model group were observed in DBA/1 and C57BL/6 mice. DBA/1 mice exhibited greater hyperalgesia and higher levels of inflammatory mediators. miR-143-3p expression in the blood and the dorsal root ganglion (DRG) were detected, and low miR-143-3p expression was demonstrated in the blood and DRG tissue of CIA mice. The target genes of miR-143 were predicted and analyzed. A total of 1,305 genes were predicted and 55 pain-associated genes were obtained. Prostaglandin-endoperoxide synthase 2 (Ptgs2), MAS related GPR family member E (Mrgpre), prostaglandin D2 receptor and Tnf were selected as target genes of miR-143. DRG cells were cultured and transfected with miR-143-3p inhibitor or mimic. The expression of Mrgpre, Ptgs2 and Tnf was significantly inhibited following miR-143-3p mimic transfection, while the expression of Mrgpre, Ptgs2 and Tnf was increased following inhibitor transfection. Additionally, the expression of pain-associated genes in the DRG of mice was investigated and the expression of Ptgs2, Mrgpre and Tnf in the DRG of CIA mice was also significantly upregulated. These results revealed that CIA mice exhibited marked hyperalgesia and high levels of inflammatory pain mediators. Low expression of miR-143-3p negatively regulated the pain-associated target genes, including Mrgpre, Ptgs2 and Tnf, thereby affecting chronic inflammatory pain and neuropathic pain in RA.

Introduction

Rheumatoid arthritis (RA) is a common inflammatory autoimmune disease and the associated inflammation has been extensively studied. RA patients suffer from pain (1-3), which affects their daily lives (4). RA pain is frequently characterized by neuropathic pain. The dorsal root ganglion (DRG) is a place where primary sensory neurons accumulate in the pain conduction pathway (5), which may selectively perceive noxious stimulation and potential tissue damage. The sensory neuron sensitization of the DRG is one of the principal mechanisms underlying pain responses that are affected by the interaction of inflammatory cells with neurons in the DRG and the central nervous system. In addition, the release of inflammatory mediators (e.g., various prostaglandins, leukotrienes and interleukins) serves a crucial role in mediating the chronic pain of RA. The inflammatory mediators lead to peripheral sensitization of sensory nerves, and alterations in nerve growth and gene expression. Therefore, RA pain originates from joint inflammation and pain signal transduction processes, with complex regulatory mechanisms. It is, therefore, of great interest to identify whether there is a modulator that simultaneously influences the release of inflammatory pain mediators and pain conduction.

Previously, microRNAs (miRs) have been associated with certain pathological mechanisms for chronic inflammatory pain and neuropathic pain (6,7). The expression of miRs is significantly altered in different nerve tissues of previous pain models. In addition, selective accumulation or deletion of miRs in local synapses can regulate gene expression, protein synthesis and inflammatory pain-associated transcription factors (8), being

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closely associated with the occurrence and maintenance of pain. The authors' previous studies have also confirmed abnormalities in miR expression and alterations in miR-143 expression altered in RA patients and animal models (9,10). Certain reports also demonstrated that miR-143 had an association with inflammatory pain responses. For example, the expression of hsa-miR-143-3p is reduced in patients with fibromyalgia (11); miR-143 degrades cyclooxygenase (COX)-2 mRNA and regulates pain mediator production in pancreatic cancer (12). Tam *et al* (13) demonstrated that the expression levels of miR-143 were significantly lower in DRGs ipsilateral to complete Freund's adjuvant (CFA) injection. Therefore, the present study aimed to investigate the role of miR-143 in the complex mechanism of RA pain and to identify key pain targets for regulating pain responses.

In the present study, collagen-induced arthritis (CIA) was first compared by observing pain responses and detecting the levels of inflammatory pain mediators in the DBA/1 and C57BL/6 mice. miR-143 expression in the peripheral blood and DRG of CIA mice was further investigated, and the target genes that regulated RA pain responses were clarified. The results may provide novel insights into the development of strategies to prevent or alleviate RA pain.

Materials and methods

Animals. A total of 18 DBA/1 and 12 C57BL/6 male mice aged 6-8 weeks (16-20 g) were provided by the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China). The experiment was performed in the Experimental Animal Center of Nanjing University of Chinese Medicine (Jiangsu, China). Mice were housed in a temperature- (22±3˚C) and humidity-controlled (40-70%) animal room under a 12-h light/dark cycle, with free access to food and water. All experimental protocols performed in this study were approved by the Ethics Review Committee for Animal Experimentation of Nanjing University of Chinese Medicine and were in accordance with the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals (14).

Establishment of the DBA/1 or C57BL/6 mouse model of CIA (15). Bovine type II collagen (CII; Chondrex, Inc., Redmond, WA, USA) was dissolved in 0.01 mol/l acetic acid into a 2 g/l solution and shaken overnight at 4˚C. The solution was mixed with CFA (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at a ratio of 1:1 and emulsified. A total of 12 DBA/1 or C57BL/6 mice were separately divided into two groups (n=6). In the model group, a mixed emulsion (100 μl) of CII and CFA was intradermally injected at the tail root of mice for primary immunization, and this day was recorded as d1. On d21, CII was mixed evenly with incomplete Freund's adjuvant (Sigma-Aldrich; Merck KGaA) at a ratio of 1:1, 100 μl of which was injected at the tail root to boost immunization. The mice in the control group were intradermally injected with normal saline at the tail root on d1 and d21, respectively.

Detection of inflammatory responses and pain mediator release in DBA/1 or C57BL/6 mice with CIA. The paw thickness of DBA/1 mice was measured on d21, d28, d35, d41 and d49, and the mice were sacrificed by anesthesia on d49. The paw thickness of C57BL/6 mice was measured on d5, d12, d19, d26, d33, d40, d47 and d54, and the mice were sacrificed by anesthesia on d54. The sera of all mice were collected and the levels of tumor necrosis factor (TNF)-α and prostaglandin (PG)E_2 were measured using mouse TNF-α (cat. no. YY02868B) and PGE_2 ELISA kits (cat. no. YY02798B; Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China).

Detection of pain responses in DBA/1 or C57BL/6 mice with CIA. Pain responses, including the mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) were measured on d20, d24, d30, d36, d42 and d48 in DBA/1 mice, and on d5, d12, d19, d26, d33, d40, d47 and d54 in C57BL/6 mice. The tests were performed as follows. The experimental environment was kept quiet. The mice were placed in a plexiglass box with metal mesh bottom. Following >30 min of adaptation, the experiment was begun when the exploration activities had stopped. Von Frey filaments (0.008-4.0 g) were used to stimulate the mouse paw vertically into a slight S shape. Obvious paw withdrawal, lifting or licking was regarded as a positive response, and no response was negative. MWT was measured and calculated using the 'up and down' method described by Chaplan *et al* (16).

Under the same conditions described above, the mouse paw was irradiated with a radiation source using a 37370 thermal plantar analgesia instrument and the avoidance time, from the initiation of irradiation to leg lifting and paw withdrawal, was recorded. Each mouse was tested three times with a 5-min interval, and the average was recorded as TWL.

Detection of miR-143-3p expression in the peripheral blood and DRG using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. A total of 6 DBA/1 mice were equally divided into a control group and a CIA group, and the CIA model was established with the method described above. Peripheral blood and DRG tissues were obtained from the control and CIA mice on d49, from which the RNA was extracted with ice-cold TRIzol® (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The RNA concentration and purity were spectrophotometrically determined at 260 and 280 nm. cDNA was synthesized using a Transcriptor First-Strand cDNA Synthesis kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). The reverse transcription reaction was performed under the following conditions: 16˚C for 30 min, 42˚C for 40 min and 85˚C for 5 min. The target miR of each sample and the internal reference (mmu-miR-425-5p) were detected using an Applied Biosystems detection instrument (Thermo Fisher Scientific, Inc.). mmu-miR-425-5p was used for normalization. The SYBR® Green qPCR Master Mix (Arraystar Inc, Rockville, MD, USA) was used and the PCR was performed using the following cycling conditions: 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 sec and 60˚C for 60 sec. The data were analyzed using the $2^{-\Delta\Delta Cq}$ method (17). A total of three replicate wells were set up and the results were averaged. The primers were as follows: mmu-miR-143-3p gene specific primer (GSP), 5'-GGG ATGAGATGAAGCACT-3' and reverse (R), 5'-TGCGTGTCG TGGAGTC-3'; mmu-miR-425-5p GSP, 5'-GGGGAATGACAC GATCACTC-3' and R, 5'-GTGCGTGTCGTGGAGTCG-3'.

Bioinformatics analysis of pain-associated target genes of miR-143. The target genes of miR-143 were predicted using bioinformatics databases, including miRanda (http://www.mirbase.

org), miRDB v. 4.0 (http://mirdb.org/miRDB), TargetScan v. 6.2 (http://www.targetscan.org), miRWalk v. 2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2) and RNA22 v. 2 (https://cm.jefferson.edu/rna22/Interactive). The target genes of the three intersects were selected to minimize the false positive rate. All the target genes were analyzed by Cytoscape software v. 3.5.0 (http://www.cytoscape.org/) to obtain the microRNA-gene regulatory network. Using the gene database from the Pain Genetics Lab (http://www.psych.mcgill.ca/labs/mogillab/pain-genetics/lab/) of McGill University (Montreal, Canada), the predicted target genes of miR-143 were analyzed comprehensively to determine potential pain-associated genes.

Gene ontology (GO) analysis and pathway analysis of miR-143 target genes. GO functional annotation enrichment analysis of the predicted miR-143 target genes was conducted using the GO database (http://www.geneontology.org), including cell components and molecular function. Using the Kyoto Encyclopedia of Gene and Genomes (KEGG) database (http://www.genome.jp/kegg) in the DAVID database, biological pathway enrichment analysis was performed on the predicted target genes.

Isolation, culture and transfection of DRG cells. In an ice bath, the spinal canal of a normal mouse was cut open under a dissecting microscope to remove the spinal cord and other nerve fibers to isolate the DRG. Subsequently, 1 ml mixed digestive enzyme was added, digestion was performed in a 37˚C water bath for 25-30 min and the resulting tissue was pipetted repeatedly into a single cell suspension. The supernatant was discarded following centrifugation (4˚C, 12,000 x g, 15 min) and 1 ml DH10 medium (WISENT Inc., Quebec, QC, Canada) and 1% nerve growth factor (NGF; R&D Systems, Inc., Minneapolis, MN, USA) were added to the precipitate. The cell suspension was pipetted into a single cell suspension and grown in a pre-coated petri dish. When the density of DRG cells reached 50-60% confluence, the cell culture medium was replaced prior to transfection.

The mmu-miR-143-3p sequence (Shanghai GenePharma Co., Ltd., Shanghai, China) was dissolved into a 10 μM stock solution, aliquoted and stored at-20˚C. When the density of DRG cells reached 50-60%, the culture medium was refreshed prior to transfection and 2 ml antibiotic-free DH10 medium was added into each dish. A total of 3 μl miR mimic, inhibitor or miR negative control (NC) (Shanghai GenePharma Co., Ltd.) was mixed gently with 150 μl Opti-MEM reduced serum medium (Gibco; Thermo Fisher Scientific, Inc.). A total of 9 μl Lipofectamine® RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) was diluted with 150 μl Opti-MEM reduced serum medium. Diluted Lipofectamine® RNAiMAX was mixed gently with diluted miR. miR-143 mimic NC-FAM (carboxyfluorescein): Sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. miR-143 mimic: Sense, 5'-UGAGAUGAAGCACUGUAGCUC-3' and antisense, 5'-GCUACAGUGCUUCAUCUCAUU-3'. miR-143 inhibitor: Sense, 5'-GAGCUACAGUGCUUCAUCUCA-3'. miR-143 inhibitor NC: Sense, 5'-CAGUACUUUUGUGUAGUACAA-3'. Following incubation at room temperature for 5 min, the mixture was added into the dish containing the DRG cells and culture medium, mixed by gentle agitation and cultured in a CO_2 incubator at 37˚C. The cells were observed using fluorescence microscopy at 6, 12, 24, 48 and 72 h following transfection to determine the suitable transfection conditions.

Detection of the expression of miR-143-3p target genes in transfected DRG cells and DRG tissues of CIA mice. DRG cells were cultured and transfected with the method described above. miR-143-3p mimic, inhibitor and miR NCs were transfected into DRG cells for 48 h. RNA was extracted from DRG cells and the expression of mmu-miR-143, MAS related GPR family member E (Mrgpre), prostaglandin endoperoxide synthase 2 (Ptgs2, also known as COX-2), prostaglandin D2 receptor (Ptgdr) and Tnf were detected using qPCR.

A total of 6 DBA/1 mice were divided into a control group and a CIA group. The CIA model was established by the method described above. DRG tissues were obtained from the control and CIA mice on d49, from which RNA was extracted. The expression of Mrgpre, Ptgs2 and Tnf were detected using RT-qPCR, as described above.

The primers were as follows: Mrgpre forward (F), 5'-CAGAACCACCCGTAGGCAAAT-3' and R, 5'-TTCCAGGTCACCGTCCATCAC-3'; Ptgs2 F, 5'-CAGATGACTGCCCAACTCCCA-3' and R, 5'-GTGAACCCAGGTCCTCGCTTA-3'; Ptgdr F, 5'-AACACCGTCTCACTGTAGGCTT-3' and R, 5'-CTGGTTTCCCAACTCATTTCTC-3'; Tnf F, 5'-GAGTCCGGGCAGGTCTACTTT-3' and R, 5'-CAGGTCACTGTCCCAGCATCT-3'; U6 F, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and R, 5'-CGCTTCACGAATTTGCGTGTCAT-3'; and GAPDH F, 5'-CACTGAGCAAGAGAGGCCCTAT-3' and R, 5'-GCAGCGAACTTTATTGATGGTATT-3'.

Data processing and statistics. The experiments were repeated three times. Experimental data are expressed as the means ± standard deviation. T-test was used for the comparison of two groups and analysis of variance followed by Tukey's test was used for the comparison of multiple groups. Data processing was performed using the SPSS v. 13.0 statistical software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Comparison of inflammatory pain responses and pain mediator release in DBA/1 or C57BL/6 mice with CIA. DBA/1 and C57BL/6 mouse models of CIA were constructed, and the alterations of CIA-associated indices were investigated. The forelimb and hindlimb became red and swollen from d24 (DBA/1 mice) and d33 (C57BL/6 mice), and their paws and ankle joints were also notably affected on d28 (DBA/1 mice) and d40 (C57BL/6 mice). The paw thickness markedly increased with the progression of arthritis in the CIA group compared with the control group ($P<0.01$; Fig. 1A and B).

MWT and TWL, which indicate pain responses, were also detected. For DBA/1 mice, the TWL of the CIA model group decreased significantly from d24 ($P<0.01$; Fig. 1C) and gradually recovered from d42, and MWT dropped significantly from d30 ($P<0.01$; Fig. 1E). For C57BL/6 mice, TWL was slightly lower compared with the control group ($P>0.05$; Fig. 1D) and the MWT of the CIA model group significantly decreased ($P<0.05$; Fig. 1F).

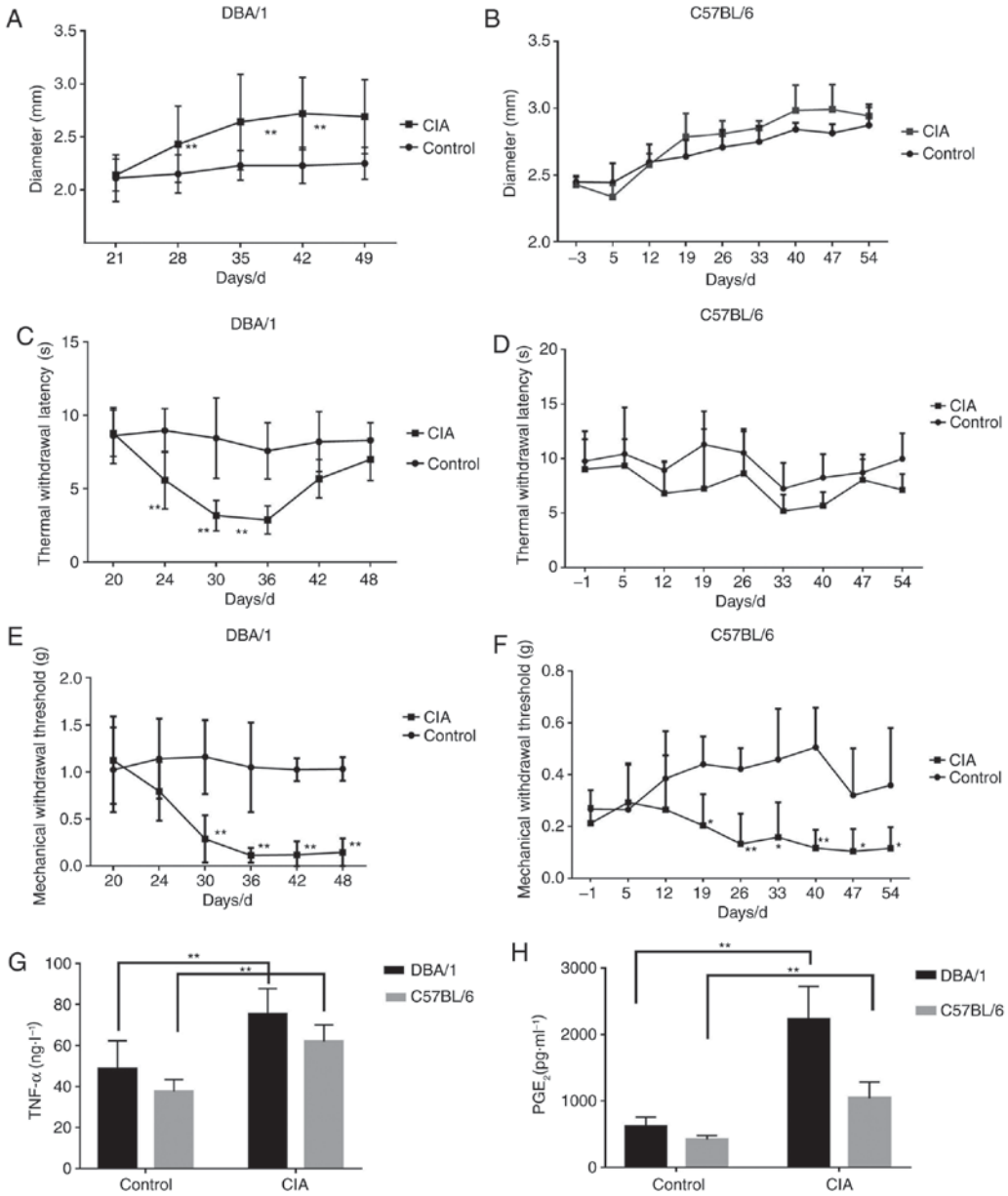

Figure 1. Inflammatory reactions and pain responses in DBA/1 or C57BL/6 mice with CIA. Paw thickness was measured between d21 and d49. TWL and MWT were detected between d20 and d48. The levels of TNF-α and PGE_2 in the sera were determined by ELISA on d49. (A) Footpad diameter of DBA/1 mice. (B) Footpad diameter of C57BL/6 mice. (C) TWL of DBA/1 mice. (D) TWL of C57BL/6 mice. (E) MWT of DBA/1 mice. (F) MWT of C57BL/6 mice. (G) TNF-α levels in the sera. (H) PGE_2 levels in the sera. *P<0.05, **P<0.01 vs. respective control. TWL, thermal withdrawal latency; MWT, mechanical withdrawal threshold; TNF, tumor necrosis factor; PGE_2, prostaglandin E_2; CIA, collagen-induced arthritis.

Additionally, the levels of inflammatory pain mediators, including TNF-α and PGE_2, significantly increased in the CIA model group compared with the control group for the two strains of mice (P<0.01; Fig. 1G and H).

The results indicated that DBA/1 mice underwent more marked hyperalgesia and had increased levels of inflammatory mediators compared with the C57BL/6 mice, thus they were selected for the following experiments.

miR-143-3p expression in the peripheral blood and DRG. In the authors' previous studies, miRs in CIA mice and normal mice were screened, revealing that miR-143-3p was expressed at a low level (9,10). In the present study, miR-143-3p expression in the peripheral blood and DRG was detected by RT-qPCR. The relative expression of miR-143-3p in the peripheral blood of CIA mice was significantly lower compared with the normal control mice (P<0.05; Fig. 2A). In addition, miR-143-3p expression in the DRG tissue of the CIA mice was also significantly downregulated (P<0.05; Fig. 2B). These results further confirmed that low miR-143-3p expression was an important feature of the CIA mouse model.

Prediction of pain-associated target genes of miR-143. The target genes of miR-143 were predicted and analyzed. The miR-target gene regulatory network is exhibited in Fig. 3. A total of 1,305 genes, including mitogen-activated protein kinase (MAPK)7, MAPK3, COX2, matrix metalloproteinase and TNF, were predicted to be the target genes of miR-143. Using the gene database of the Pain Genetics Lab,

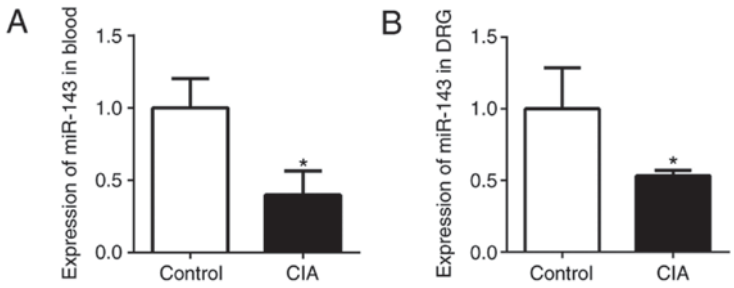

Figure 2. miR-143-3p expression measured by reverse transcription quantitative polymerase chain reaction in DBA/1 mice. miR-143-3p expression in (A) the peripheral blood and (B) DRG of mice. *P<0.05 vs. control. CIA, collagen-induced arthritis; miR, microRNA; DRG, dorsal root ganglion.

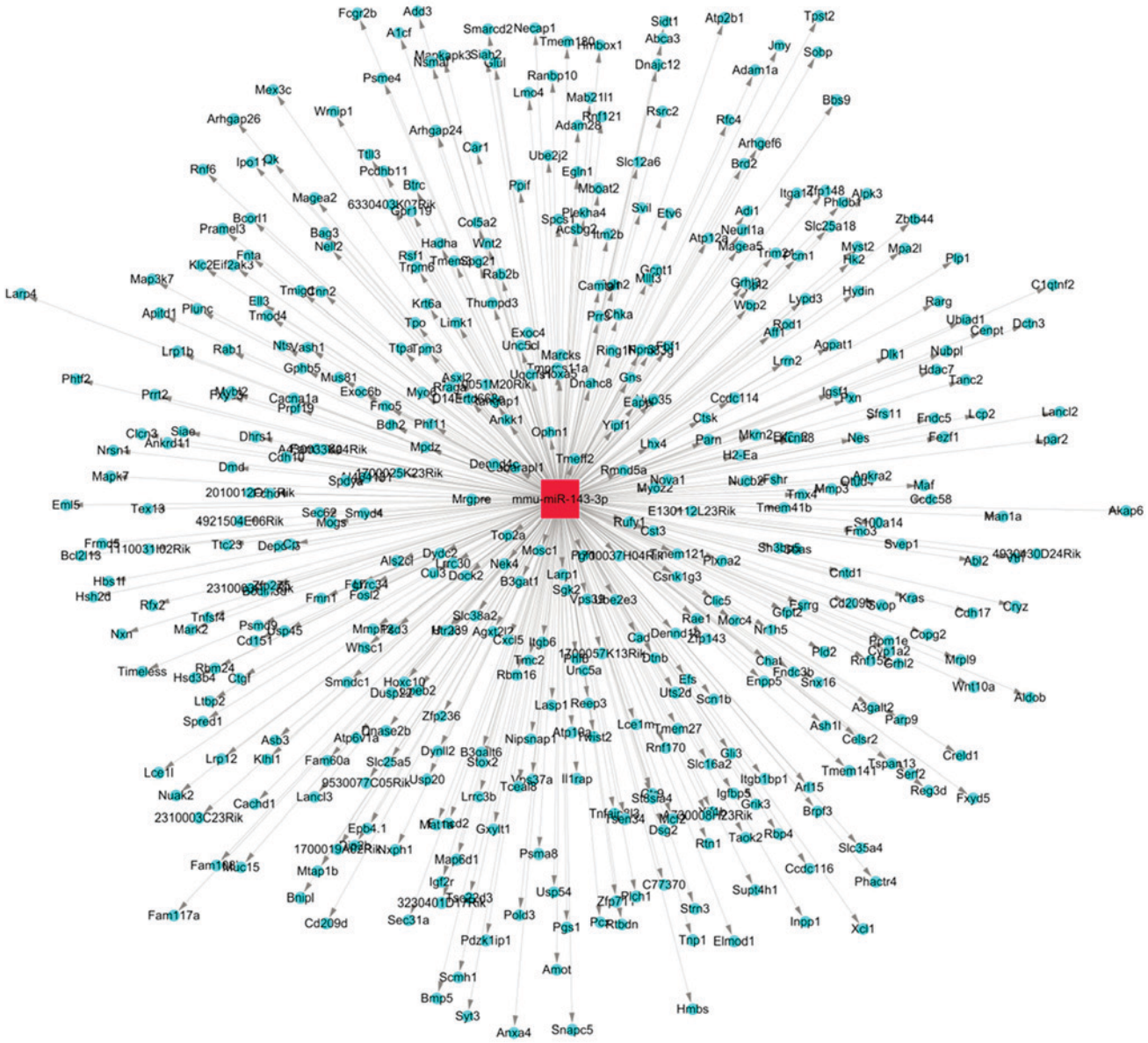

Figure 3. miR-143 target gene regulatory network. The red square represents the miR, the blue circles represent the target genes and the lines with grey arrowheads indicate their interactions. miR, microRNA.

55 pain-associated genes were obtained as potential target genes of miR-143 (Table I).

The results of the GO analysis demonstrated that the genes were associated with 'Golgi apparatus', 'plasma membrane', 'axon', 'neuron projection', 'vesicle', 'cytoplasmic vesicle' and 'endoplasmic reticulum'. The results also demonstrated that the genes were associated with various molecular functions (Fig. 4A), including 'ribonucleotide binding', 'purine ribonucleotide binding', 'ATP binding', 'solute: sodium symporter activity' and 'ion binding'.

Table I. Potential pain-associated target genes of miR-143.

Gene	Protein name	Protein acronym
Accn1	Amiloride-sensitive cation channel 1, neuronal (degenerin)	ASIC2
Adcy1	Adenylatecyclase 1	AC1
Adra1d	Adrenergic receptor, α1d	a1D-AR
Adra2c	Adrenergic receptor, α2c	a2C-AR
Adrbk2	Adrenergic receptor kinase, β2	GRK3
Agtr2	Angiotensin II receptor, type 2	AT2R
Bace1	β-site APP cleaving enzyme 1	-
Cacnb3	Calcium channel, voltage-dependent, β3 subunit	b3
Calb1	Calbindin1	Calb-1
Ccr5	Chemokine (C-C motif) receptor 5	CCR5
Cd274	CD274 antigen	B7-H1
Cd40	CD40 antigen	CD40
Chrna4	Cholinergic receptor, nicotinic, α polypeptide 4	Acra4
Clock	Circadian locomoter output cycles kaput	CLOCK
Cnr1	Cannabinoid receptor 1 (brain)	CB1
Cxcr3	Chemokine (C-C-C motif) receptor 3	CXCR3
Dicer1	Dcr-1 homolog (Drosophila)	-
Dlg2	Discs, large homolog 2 (Drosophila)	-
Drd1a	Dopamine receptor D1A	DRD1
Foxn1	Forkhead box N1	nu
Gabbr1	γ-aminobutyric acid (GABA-B) receptor, 1	GABA-B(1)
Gad2	Glutamic acid decarboxylase 2	GAD65
Gja1	Gap junction protein, α1	Cx43
Gria1	Glutamate receptor, ionotropic, AMPA1 (α1)	GluR-1
Ifngr1	Interferon γ receptor 1	IFNgR
Ikbke	Inhibitor of κB kinase ε	IKKepsilon
Il1rap	Interleukin 1 receptor accessory protein	IL-1RAcP
Kcna1	Potassium voltage-gated channel, shaker-related subfamily, member 1	Kv1.1
L1cam	L1 cell adhesion molecule	L1
Mgll	Monoglyceride lipase	MAGL
Mmp24	Matrix metalloproteinase 24	MT5-MMP
Mrgpre	MAS related GPR, member E	MrgE
Nf1	Neurofibromatosis 1	Nf-1
Ngfr	Nerve growth factor receptor (TNFR superfamily, member 16)	p75
Nlgn2	Neuroligin 2	NL2
Nptx1	Neuronal pentraxin 1	NP1
Npy	Neuropeptide Y	NPY
Nts	Neurotensin	NT
Ntsr1	Neurotensin receptor 1	NT1R
Pcsk2	Proprotein convertase subtilisin/kexin type 2	PC2
Pik3cg	Phosphoinositide-3-kinase, catalytic, γ polypeptide	PI3Kg
Plp1	Proteolipid protein (myelin) 1	Plp
Ppp1r9b	Protein phosphatase 1, regulatory subunit 9B	-
Ptgdr	Prostaglandin D receptor	DP
Ptgfr	Prostaglandin F receptor	FP
Ptgs2	Prostaglandin-endoperoxide synthase 2	COX-2
Rabggta	Rab geranylgeranyl transferase, a subunit	gm
Slc12a6	Solute carrier family 12, member 6	KCC3
Slc6a2	Solute carrier family 6 (neurotransmitter transporter, noradrenalin), member 2	NET
Slc6a4	Solute carrier family 6, member 4	5-HTT
Stx1a	Syntaxin 1A (brain)	HPC-1
Tacr1	Tachykinin receptor 1	NK1R

Table I. Continued.

Gene	Protein name	Protein acronym
Tnf	Tumor necrosis factor	TNF-α
Vip	Vasoactive intestinal polypeptide	VIP

CD, cluster of differentiation; miR, microRNA.

The results of the KEGG analysis demonstrated that miR-143 was involved in the regulation of multiple pathways and its target genes were significantly enriched in 'VEGF signaling pathway', 'T cell receptor signaling pathway', 'MAPK signaling pathway', 'neurotrophin signaling pathway', 'pathways in cancer' and other signaling pathways (P<0.01).

Following this, Ptgs2, Mrgpre, Ptgdr and Tnf were selected as associated target genes for further verification, based on the results of the GO and KEGG analysis and the factors in RA inflammatory pain.

Effects of miR-143 on pain-associated target genes. To determine the association between miR-143 and target genes, DRG cells were cultured and transfected with Lipofectamine RNAiMAX transfection reagent and the miR-143-3p mimic NC-FAM. The transfected cells were observed using microscopy at 6, 12, 24, 48 and 72 h. As demonstrated in Fig. 5A and B, DRG cells were successfully transfected, and the transfection efficiency peaked (~70%) at 48 h. miR-143-3p expression significantly decreased following miR-143 inhibitor transfection for 48 h (P<0.01), whereas it increased 3.5-fold following miR-143 mimic transfection (P<0.01; Fig. 5C and D), verifying the effects of inhibitor or mimic transfection.

Subsequently, the expression of Mrgpre, Ptgs2, Ptgdr and Tnf in DRG cells was detected. As illustrated in Fig. 5E-H, the expression of Mrgpre, Ptgs2 and Tnf was significantly inhibited (P<0.05) following miR-143-3p mimic transfection, although it was significantly promoted following miR-143-3p inhibitor transfection (P<0.05). The mimic and inhibitor transfections did not markedly affect Ptgdr expression.

Expression of pain-associated genes in the DRG of CIA mice. The expression of Ptgs2, Mrgpre and Tnf in the DRG of CIA and control mice was also detected by RT-qPCR. Compared with the control mice, the expression of Ptgs2, Mrgpre and Tnf in the DRG of CIA mice was significantly upregulated (P<0.05; Fig. 6). The expression of miR-143-3p was negatively associated with the expression of Mrgpre, Ptgs2 and Tnf in CIA mice, being consistent with the results of the *in vitro* experiments.

Discussion

The pain of RA patients is associated with multiple complex mechanisms, including inflammation, peripheral and central pain processing, and joint structure damage (18). Therefore, it may be useful for effective treatment to investigate the mechanisms of pain. In this study, the role of miR-143 in RA pain was investigated and the mechanism by which miR-143 may regulate the pain of CIA is described for the first time, to the best of the authors' knowledge.

The CIA model has a similar pathological mechanism to that of human RA (19) and it is the most widely used mouse model for RA (20). CII is the principal constituent collagen form in articular cartilage, and immunoreactivity to CII maybe identified in certain patients with RA. Immunization with CII leads to the development of severe polyarticular arthritis mediated by immune mechanisms. The autoimmune response involves CII-specific T cells and B cells and their products, proinflammatory cytokines and anti-CII antibodies (21). Thus, the CIA model may mimic the responses of RA pain more accurately compared with other pain models. Considering that different strains of mice may have a number of different symptoms, two strains of mice were tested, and it was demonstrated that they were subjected to apparent CIA. MWT and TWL are the common indices that reflect pain sensitivity to mechanical and thermal stimuli. In the present study, the MWT and TWL values of DBA/1 and C57BL/6 CIA mice decreased, demonstrating that the CIA animal models exhibited pain behaviors. Furthermore, DBA/1 CIA mice underwent increased hyperalgesia compared with the C57BL/6 mice. Also, the levels of PGE_2 and TNF-α increased in the CIA mice. The results of the present study also confirmed the increase in PGE_2 and TNF-α levels in CIA mice. As a well-known mediator of inflammation and pain, PGE_2 serves a key role in the occurrence and maintenance of RA pain and hyperalgesia (22). TNF-α, an essential inflammatory factor for RA (23), is able to induce the release of bradykinin, promote the release of substance P from nerve terminal receptors and accelerate the synthesis of PGE_2 through COX activation (24,25). Therefore, increasing inflammatory mediators may be one of the mechanisms underlying the pain response. DBA/1 CIA mice exhibited increased levels of inflammatory mediators compared with C57BL/6 mice. Accordingly, DBA/1 mice were selected to construct the CIA model for further investigation.

Low-level expression of miR-143 in the peripheral blood of patients with RA and CIA mice has been previously demonstrated (9). In addition, miR-143 has been associated with inflammatory pain responses (11,12). In the present study, RT-qPCR demonstrated that miR-143 was expressed at a low level in the peripheral blood of CIA mice. Considering the role of the DRG in pain conduction, miR-143 expression was also measured, and the results demonstrated that miR-143 may be an important modulator of the responses to CIA pain.

Bioinformatics analysis also revealed that certain potential target genes of miR-143 were pain-associated and others were associated with the production of inflammatory mediators. As suggested by GO and KEGG analysis, the cytokines may be synthesized in the Golgi apparatus and may be transported in a

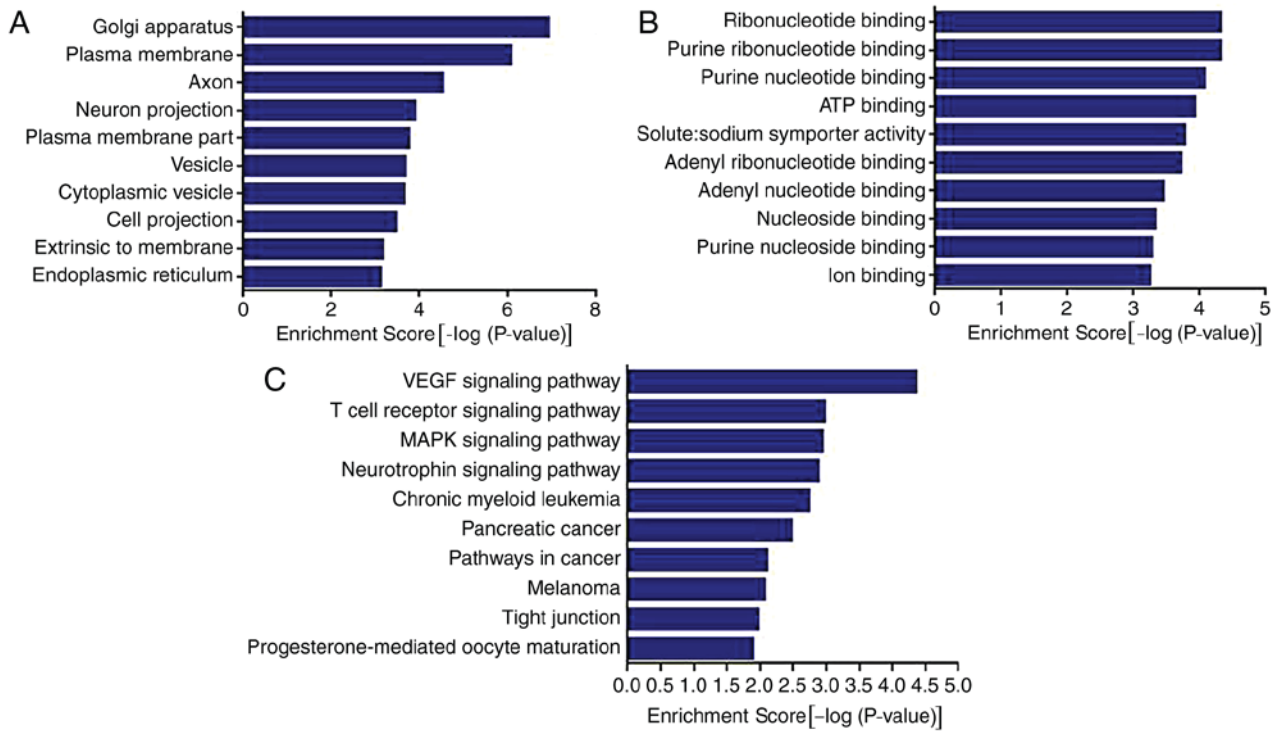

Figure 4. GO and KEGG analysis of miR-143 target pain-associated genes. (A) GO analysis of cell components. (B) GO analysis of molecular functions. (C) KEGG analysis of miR-143. GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; miR, microRNA; VEGF, vascular endothelial growth factor; MAPK, mitogen-activated protein kinase; ATP, adenosine triphosphate.

soluble form via the endoplasmic reticulum. Alternatively, they may be present in a membrane-bound form or processed into an intracellular form and transported in the cell. Given that Ptgs2, Ptgdr and Tnf have been closely associated with the release of inflammatory pain mediators in RA, they may be the miR-143-3p target genes for pain regulation. In addition, pain conduction is also worthy of attention according to the results of the GO analysis. For example, target-gene associated axons, the output channels of the neurons, are the principal signal transmission channels in the nervous system. The analysis of the molecular functions also indicated that miR-143 may regulate gene expression by influencing nucleotide binding. It may also regulate the selective transport of sodium ions, which maintain the cell excitability and signal transduction, and affect peripheral and central nervous system sensitization. miR-143 may also influence adenosine triphosphate (ATP) release and its combination with the ATP receptor in the primary afferent, and subsequently modulate the excitement of the DRG nociceptive neurons. Among the potential genes, Mrgpre is selectively highly expressed in the DRG and small neurons of the trigeminal ganglion, which are closely associated with pain conduction, being involved in the onset of chronic pain and hyperalgesia (26). Therefore, Mrgpre may be the target gene of miR-143-3p.

To further clarify the target genes of miR-143-3p in CIA pain responses, mouse DRG cells were cultured and transfected with an miR-143-3p inhibitor or mimic. The expression of certain potential genes in the transfected cells was compared, and it was demonstrated that the expression of Mrgpre, Ptgs2 and Tnf was negatively associated with miR-143-3p, although Ptgdr expression was not notably affected. Therefore, Mrgpre, Ptgs2 and Tnf may be the target genes of miR-143-3p. The results of the *in vivo* experiment were similar. In detail, the expression of Mrgpre, Ptgs2 and Tnf increased in the DRG of CIA mice. Low miR-143-3p expression in RA may induce upregulation of Mrgpre in nervous tissues, therefore affecting the onset of hyperalgesia and pain conduction. In addition, Ptgs2 and Tnf expression were also upregulated. Ptgs2 in DRG cells may facilitate the synthesis of PGE_2 (27), activate the corresponding receptors in the DRG and a variety of cellular signaling pathways, augment the sensitivity of nociceptors to pain stimulation, and finally cause hyperalgesia and allodynia (18,28). Tnf mRNA may further regulate the release of TNF-α and subsequently activate COX to enhance the regulation of PGEs (23,25). Pham *et al* (12) also reported that miR-143 degraded COX-2 mRNA and regulated PGE production. Therefore, miR-143-3p may modulate the levels of inflammatory algogenic substances (PGE_2 and TNF-α) by regulating its target genes. Similarly, the increasing levels of PGE_2 and TNF-α in CIA mice concurred with the above analysis. Taken together, miR-143-3p regulated pain transduction and the release of inflammatory pain mediators, thereby regulating the complex mechanisms of RA pain.

In conclusion, CIA mice were subjected to hyperalgesia and the release of inflammatory pain mediators. Low expression of miR-143-3p negatively regulated pain-associated target genes, including Mrgpre, Ptgs2 and Tnf, thereby affecting the chronic inflammatory pain and neuropathic pain of RA. Seeking drugs that regulate miR-143 may be useful for the treatment of RA pain.

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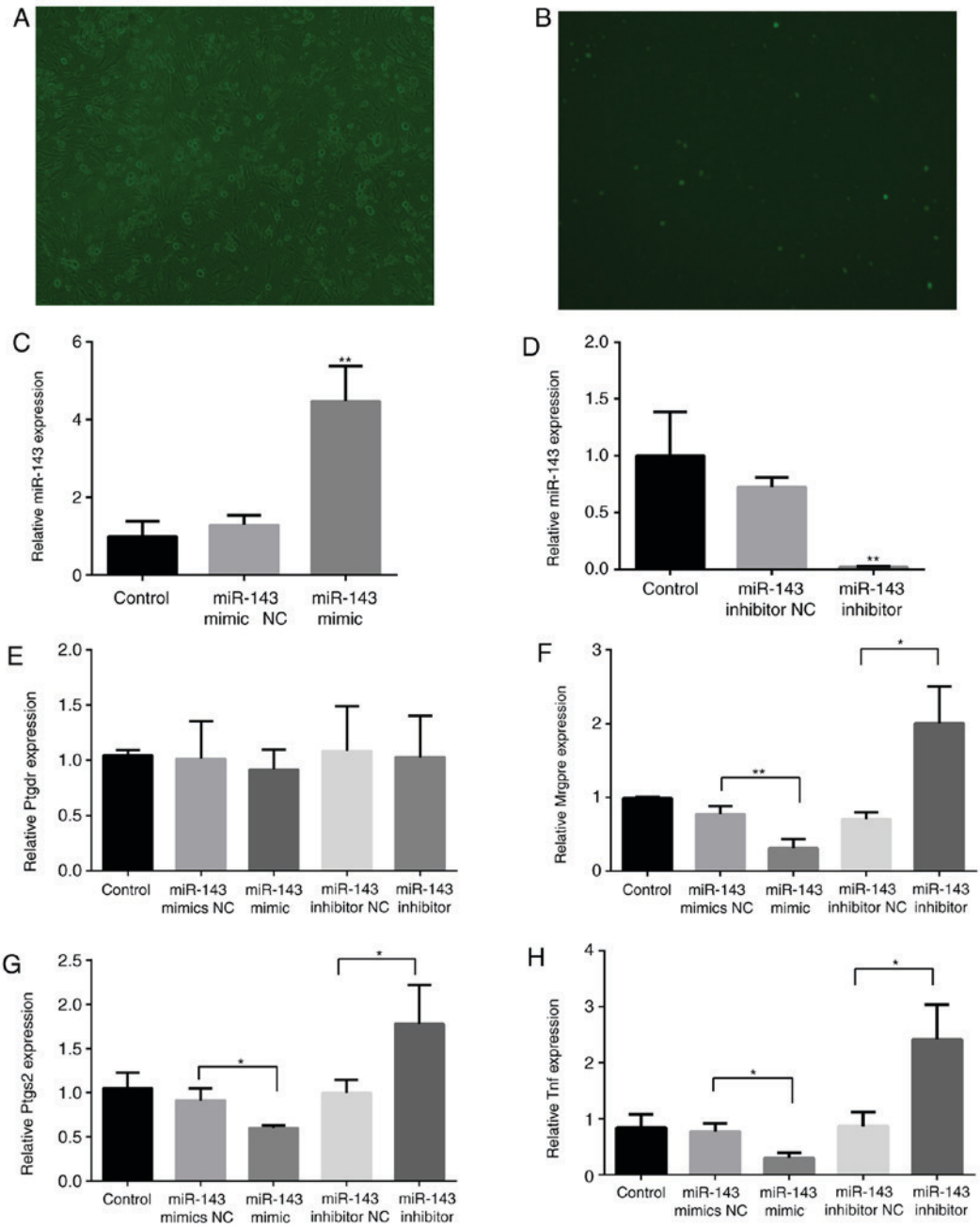

Figure 5. Effects of miR-143 on pain-associated target genes. Mouse DRG cells were transfected with Lipofectamine RNAiMAX transfection reagent and the miR-143-3p mimic NC-carboxyfluorescein. (A) Bright field (magnification, x100). (B) Fluorescence microscopy (magnification, x100). RNA was extracted from the DRG cells 48 h following transfection with an miR-143-3p inhibitor or mimic. (C) miR-143-3p expression following transfection with the mimic. (D) miR-143-3p expression following transfection with the inhibitor. (E) Ptgdr expression, (F) Mrgpre expression, (G) Ptgs2 expression and (H) Tnf expression. *P<0.05, **P<0.01 vs. NC group. miR, microRNA; TNF, tumor necrosis factor; DRG, dorsal root ganglion; Mrgpre, MAS related GPR family member E; Ptgdr, prostaglandin D2 receptor; Ptgs2, prostaglandin-endoperoxide synthase 2; NC, negative control.

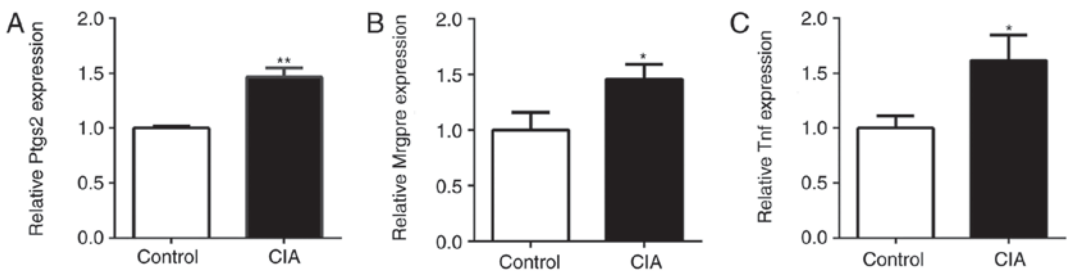

Figure 6. Expression of pain-associated genes in the dorsal root ganglia of CIA mice. (A) Ptgs2 expression, (B) Mrgpre expression and (C) Tnf expression. *P<0.05, **P<0.01 vs. control. Mrgpre, MAS related GPR family member E; Ptgs2, prostaglandin-endoperoxide synthase 2; TNF, tumor necrosis factor; CIA, collagen-induced arthritis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LLZ and XPZ conceived the project and designed the experiments. LLZ, YMZ, FYQ, CCY and DPY performed the experiments. LLZ and YMZ analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental protocols performed in this study were approved by the Ethics Review Committee for Animal Experimentation of Nanjing University of Chinese Medicine and were in accordance with the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals (14).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Heiberg T and Kvien TK: Preferences for improved health examined in 1,024 patients with rheumatoid arthritis: pain has highest priority. Arthritis Rheum 47: 391-397, 2002.
2. Walsh DA and Mcwilliams DF: Pain in rheumatoid arthritis. Curr Pain Headache Rep 16: 509-517, 2012.
3. Mcwilliams DF, Zhang W, Mansell JS, Kiely PD, Young A and Walsh DA: Predictors of change in bodily pain in early rheumatoid arthritis: An inception cohort study. Arthrit Care Res (Hoboken) 64: 1505-1513, 2012.
4. Leffler AS, Kosek E, Lerndal T, Nordmark B and Hansson P: Somatosensory perception and function of diffuse noxious inhibitory controls (DNIC) in patients suffering from rheumatoid arthritis. Eur J Pain 6: 161-176, 2002.
5. Gold MS and Gebhart GF: Nociceptor sensitization in pain pathogenesis. Nat Med 16: 1248-1257, 2010.
6. Bai G, Ambalavanar R, Wei D and Dessem D: Downregulation of selective microRNAs in trigeminal ganglion neurons following inflammatory muscle pain. Mol Pain 3: 15, 2007.
7. Aldrich BT, Frakes EP, Kasuya J, Hammond DL and Kitamoto T: Changes in expression of sensoryorgan-specific microRNAs in rat dorsal root ganglia in association with mechanical hypersensitivity induced by spinal nerve ligation. Neuroscience 164: 711-723, 2009.
8. Zhao J, Lee MC, Momin A, Cendan CM, Shepherd ST, Baker MD, Asante C, Bee L, Bethry A, Perkins JR, *et al*: Small RNAs control sodium channel expression, nociceptor excitability, and pain thresholds. J Neurosci 30: 10860-10871, 2010.
9. Zhu YM, Zhou LL, Peng XW, Geng S and Zhou XP: Study of Qingluo Tongbi Compound treating rheumatoid arthritis based on miRNA network. Chin J Immunol 32: 495-499, 2016 (In Chinese).
10. Yuan CC, Geng SA, Zhu YM, *et al*: Comparision of abnormal expression of miRNAsin peripheral blood of rheumatoid arthritis patients and osteoclasts in rat and analysis of MiRNAs. Chin J Immunol 33: 715-720, 2017 (In Chinese).
11. Cerdá-olmedo G, Mena-durán AV, Monsalve V and Oltra E: Identification of a MicroRNA Signature for the Diagnosis of Fibromyalgia. PLoS One 10: e0121903, 2015.
12. Pham H, Rodriguez CE, Donald GW, Hertzer KM, Jung XS, Chang HH, Moro A, Reber HA, Hines OJ and Eibl G: miR-143 decreases COX-2 mRNA stability and expression in pancreatic cancer cells. Biochem Biophys Res Commun 439: 6-11, 2013.
13. Tam Tam S, Bastian I, Zhou XF, Vander Hoek M, Michael MZ, Gibbins IL and Haberberger RV: MicroRNA-143 expression in dorsal root ganglion neurons. Cell Tissue Res 346: 163-173, 2011.
14. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals: Guide for the Care and Use of Laboratory Animals. Publication no. 85-23 (rev.) 8th edition. The National Academies Press 327: 963-965, Washington, DC, 2011.
15. Brand DD, Kang AH and Rosloniec EF: The mouse model of collagen-induced arthritis. Methods Mol Med 102: 295-312, 2004.
16. Chaplan SR, Bach FW, Pogrel JW, Chung JM and Yaksh TL: Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Meth 53: 55-63, 1994.
17. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
18. Schweinhardt P, Kalk N, Wartolowska K, Chessell I, Wordsworth P and Tracey I: Corrigendum to Investigation into the neural correlates of emotional augmentation of clinical pain. Neuroimage 56: 384, 2011.
19. Billiau A and Matthys P: Collagen-induced arthritis and related animal models: How much of their pathogenesis is auto-immune, how much is auto-inflammatory. Cytokine Growth F R 22: 339-344, 2011.
20. Luross JA and Williams NA: The genetic and immunopathological processes underlying collagen-induced arthritis. Immunology 103: 407-416, 2001.
21. Cook AD, Rowley MJ, Stockman A, Muirden KD and Mackay IR: Specificity of antibodies to type II collagen in early rheumatoid arthritis. J Rheumatol 21: 1186-1191, 1994.
22. Walsh DA and Mcwilliams DF: Mechanisms, impact and management of pain in rheumatoid arthritis. Nat Rev Rheumatol 10: 581-592, 2014.
23. Junger H and Sorkin LS: Nociceptive and inflammatory effects of subcutaneous TNFalpha. Pain 85: 145-151, 2000.
24. Weinblatt ME, Kremer JM, Bankhurst AD, Bulpitt KJ, Fleischmann RM, Fox RI, Jackson CG, Lange M and Burge DJ: A trial of etanercept, a recombinant tumor necrosis factor receptor:Fc fusion protein, in patients with rheumatoid arthritis receiving methotrexate. N Engl J Med 340: 253-259, 1999.
25. Boettger MK, Weber K, Grossmann D, Gajda M, Bauer R, Bär KJ, Schulz S, Voss A, Geis C, Bräuer R and Schaible HG: Spinal tumor necrosis factor α neutralization reduces peripheral inflammation and hyperalgesia and suppresses autonomic responses in experimental arthritis: A role for spinal tumor necrosis factor α during induction and maintenance of peripheral inflamma. Arthritis Rheum 62: 1308-1318, 2010.
26. Cox PJ, Pitcher T, Trim SA, Bell CH, Qin W and Kinloch RA: The effect of deletion of the orphan G- protein coupled receptor (GPCR) gene MrgE on pain-like behaviours in mice. Mol Pain 4: 2, 2008.
27. Basbaum AI, Bautista DM, Scherrer G and Julius D: Cellular and molecular mechanisms of pain. Cell 139: 267-284, 2009.
28. Julius D and Basbaum AI: Molecular Mechanisms of nociception. Nature 413: 203-210, 2001.