



■ ARTHRITIS

MicroRNA-183 attenuates osteoarthritic pain by inhibiting the *TGF α* -mediated *CCL2/CCR2* signalling axis

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Aims

MicroRNA-183 (*miR-183*) is known to play important roles in osteoarthritis (OA) pain. The aims of this study were to explore the specific functions of *miR-183* in OA pain and to investigate the underlying mechanisms.

Methods

Clinical samples were collected from patients with OA, and a mouse model of OA pain was constructed by surgically induced destabilization of the medial meniscus (DMM). Reverse transcription quantitative polymerase chain reaction was employed to measure the expression of *miR-183*, transforming growth factor α (*TGF α*), C-C motif chemokine ligand 2 (*CCL2*), proinflammatory cytokines (interleukin (IL)-6, *IL-1 β* , and tumour necrosis factor- α (*TNF- α*)), and pain-related factors (transient receptor potential vanilloid subtype-1 (*TRPV1*), voltage-gated sodium 1.3, 1.7, and 1.8 (*Nav1.3*, *Nav1.7*, and *Nav1.8*)). Expression of *miR-183* in the dorsal root ganglia (DRG) of mice was evaluated by in situ hybridization. *TGF α* , *CCL2*, and C-C chemokine receptor type 2 (*CCR2*) levels were examined by immunoblot analysis and interaction between *miR-183* and *TGF α* , determined by luciferase reporter assay. The extent of pain in mice was measured using a behavioural assay, and OA severity assessed by Safranin O and Fast Green staining. Immunofluorescent staining was conducted to examine the infiltration of macrophages in mouse DRG.

Results

miR-183 was downregulated in tissue samples from patients and mice with OA. In DMM mice, overexpression of *miR-183* inhibited the expression of proinflammatory cytokines (*IL-6*, *IL-1 β* , *TNF- α*) and pain-related factors (*TRPV1*, *Nav1.3*, *Nav1.7*, *Nav1.8*) in DRG. OA pain was relieved by *miR-183*-mediated inhibition of macrophage infiltration, and dual luciferase reporter assay demonstrated that *miR-183* directly targeted *TGF α* .

Conclusion

Our data demonstrate that *miR-183* can ameliorate OA pain by inhibiting the *TGF α -CCL2/CCR2* signalling axis, providing an excellent therapeutic target for OA treatment.

Cite this article: *Bone Joint Res* 2021;10(8):548–557.

Keywords: Osteoarthritis pain, microRNA-183, Transforming growth factor α , C-C motif chemokine ligand 2, C-C chemokine receptor 2, Inflammatory factor

Introduction

Osteoarthritis (OA) remains one of the most frequently occurring joint abnormalities, and causes an increasing social and economic burden.¹ Numerous factors contribute to the development of OA, such as body weight, diet, age, and history of trauma.^{2,3} Histological examination shows that loss of chondrocytes is a major cause of OA, as chondrocytes are critical for the formation and maintenance of the

articular cartilage.⁴ Pain is the major symptom of OA; however, current treatments for OA are inadequate in terms of therapeutic effect,^{5,6} making joint arthroplasty surgery the only effective treatment.⁷ Hence, there is an urgent need to explore the detailed mechanisms underlying OA, to facilitate development of new therapies for OA pain.

MicroRNAs (miRNAs) have critical functions in targeting several leading OA risk

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doi: 10.1302/2046-3758.108.BJR-2019-0308.R2

Bone Joint Res 2021;10(8):548–557.

factors;⁸ for example, intra-articular delivery of antago-*miR-483-5p* can disturb the development of OA by regulating matrilin 3 and tissue inhibitor of metalloproteinase 2.⁹ Moreover, *miR-126* is associated with the development of OA.¹⁰ As a miRNA highly conserved among species, *miR-183* is commonly expressed in diverse tissues and cells.¹¹ Further, *miR-183* can be applied as a powerful therapy to reduce joint pain in a mouse model of OA.¹² *miR-183* can inhibit transforming growth factor β 1 (*TGF- β 1*)-induced apoptosis of human hepatocellular carcinoma cells by targeting programmed cell death 4.¹³ Members of the *TGF- β* family are pleiotropic factors that elicit cell type-specific effects in a highly context-dependent manner and in various tissues.¹⁴ Transforming growth factor α (*TGF α*) also belongs to the *TGF- β* family and is closely associated with OA. For example, *TGF α* expression is increased in rat and human OA cartilage and modifies the phenotype of chondrocytes.¹⁵ Further, *TGF- β 1* activation is reported to contribute to OA progression,¹⁶ and studies in a murine model of OA revealed the involvement of *TGF α* .¹⁷ Interestingly, the results of a database search predicted that *miR-183* targets *TGF α* , indicating that *miR-183* may influence *TGF α* activity to regulate the pathogenesis of OA.

C-C motif chemokine ligand 2 (*CCL2*) is a member of the C-C chemokine family that is related to disease status and outcomes of OA.¹⁸ *CCL2* can specifically bind to C-C chemokine receptor type 2 (*CCR2*), which is expressed in diverse tissues,¹⁹ and both proteins have been associated with OA. For example, inhibition of *CCL2/CCR2* signalling significantly inhibits macrophage accumulation, synovitis, and cartilage damage in a mouse OA model.²⁰ In this study, we investigated the regulatory mechanisms involving *miR-183* in OA pain in relation to the *TGF α* chemokine signalling pathway.

Methods

Ethics statement. This study was approved by the Ethics Committee and Animal Care and Use committee of our hospital. All participating patients signed informed consent documents. The animal experiments were performed in compliance with the National Institute of Health's Guidelines of Laboratory Animal Care and Use in Biomedical Research, and the ARRIVE checklist was completed to show that ARRIVE guidelines were adhered to in this study.

Patient enrolment. Patients with ($n = 95$) and without ($n = 60$) OA recruited in our hospital from January 2012 to December 2016 were enrolled in the study. The 95 patients with OA included 37 males and 58 females (mean age: 58.64 years (standard deviation (SD) 5.32); mean BMI: 25.04 kg/m² (SD 4.07)), among whom 39 were diagnosed with diabetes. Patients who met all the following conditions were included: diagnosis of OA; recurrent knee pain lasting for nearly one month; radiographs indicating joint space narrowing and subchondral abnormalities, such as cysts, sclerosis, or osteophyte formation; appearance of thick and clear synovial fluid with white

blood cell count < 2,000/ml; over 40 years of age; and morning stiffness lasting < 30 minutes, with a sensation of 'grinding'. Patients who met the following conditions were excluded: allergic constitution; pregnant or lactating female; severe abnormalities affecting other organs, such as heart, brain, liver, or kidney; diagnosis of other arthritic diseases, such as rheumatoid arthritis, gouty arthritis, meniscus injury, synovitis, or ligament injury; and medicated with non-steroidal anti-inflammatory drugs (NSAIDs) in the past three months, or cortisol or hyaluronic acid preparations in the past six months. The 60 patients without OA, who served as the control group, included 23 males and 38 females (mean age: 58.72 years (SD 4.0)), had no knee joint deformity or pain in either lower limb, no history of knee joint or surrounding soft tissue injury, and no history of OA, other chronic OA, or knee joint surgery. There were no significant differences in sex or age between the two groups of patients. Joint fluid from each patient was collected and immediately placed on ice, followed by centrifugation at 3,000 \times g for ten minutes. The supernatant was snap frozen in liquid nitrogen and stored at -80°C.

Surgical destabilization of the medial meniscus to generate a mouse OA model. A total of 105 male wild-type (WT) C57BL/6 mice (mean age: 8 weeks (SD 1)) were housed in a controlled environment with food and water provided ad libitum and maintained under a 12-hour light/dark cycle. An OA mouse model was constructed by destabilization of the medial meniscus (DMM). DMM or sham surgery was performed on the right knees of male mice with a mean age of 8 \pm 1 weeks. The dissected anterior medial meniscus ligament was exposed after removal of the medial ankle joint and dissection of the anterior fat pad. The incision was closed after the knee had been washed with saline. Sham surgery was the same as DMM, except that the medial meniscus ligament was left intact.

Adenoviruses carrying *miR-183*-agomir, *miR-183*-antagomir, agomir-negative control (NC), antagomir-NC, overexpressed (oe)-*TGF α* , and oe-NC were purchased from GeneChem (China). Seven days after DMM surgery, mice were anaesthetized with 3% sodium pentobarbital (Cat. No. P3761; MilliporeSigma, USA). Thereafter, adenovirus (1×10^9 pfu/100 μ l) from each group was injected via the tail vein, with eight mice in each treatment group. The *CCR2* antagonist, RS504393 (Tocris Cookson, UK), was injected intraperitoneally (5 mg/kg), using the solvent dimethyl sulfoxide (DMSO) as a control.

Behavioural assessment of pain. Eight weeks after model establishment, weight distribution between surgical and non-surgical side paws was measured using an IITS manometer (CUSABIO Life Sciences, USA). The force of each limb exerted on a transducer plate on the floor over a given period was recorded, to measure the weight distribution placed on the two paws. Each test took over ten seconds to complete and was repeated 12 times. Mean results were expressed as the proportion of weight distribution between the limbs (left and normal vs right and arthritis). Due to the pain caused by OA, the weight on

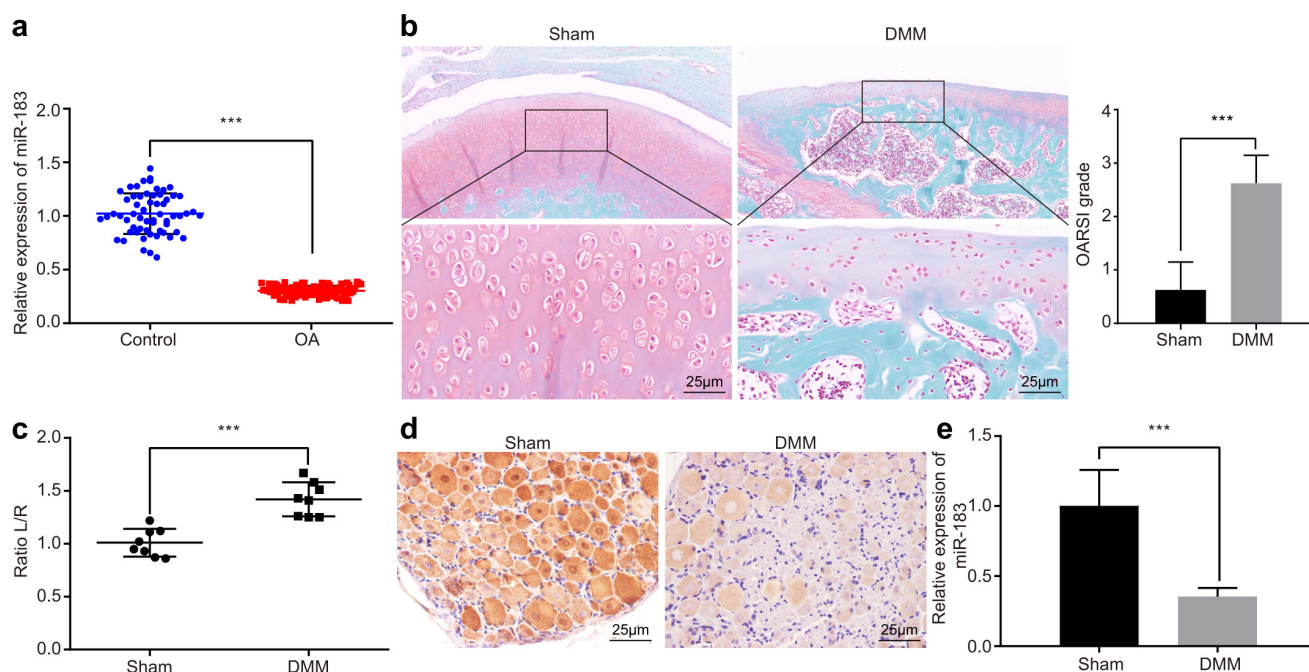


Fig. 1

microRNA-183 (*miR-183*) expression was reduced in osteoarthritis (OA) tissue samples. a) Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis of *miR-183* expression in human samples; $n = 95$ patients; $***p < 0.001$ versus the control group. b) Morphological analysis of mouse bone and joint tissue (400 \times) using Fast Green FCF (0.02%) and Safranin O (0.1%) staining, followed by Osteoarthritis Research Society International (OARSI) scoring; $n = 8$ mice in each group; $***p < 0.001$ versus the sham group. c) Evaluation of pain in mice by behavioural assessment; the weight distribution between paws on the surgical and non-surgical sides was measured using an IITS manometer ("Ratio L/R" refers to the ratio of left and normal to right and arthritis); $n = 8$ mice in each group; $***p < 0.001$ versus the sham group. d) In situ hybridization assay for *miR-183* expression in mouse L3-L5 DRG (400 \times). e) RT-qPCR analysis to evaluate *miR-183* expression in mouse L3-L5 DRG; $n = 8$ mice in each group; experiments were repeated three times; $***p < 0.001$ versus the sham group. The above results were all measurement data, and expressed as means (standard deviation (SD)). Independent-samples *t*-test was used for comparisons between two groups. DMM, destabilization of the medial meniscus.

the knee joint with surgery was less than that without surgery, resulting in a different proportion between the two paws.

Histomorphometric analysis. Eight weeks after DMM surgery, mice were euthanized using 3% sodium pentobarbital. The bone and joint tissues were collected and fixed by immersion in 4% paraformaldehyde overnight. Starting the next day, samples were decalcified in 5% methanol for seven days, then embedded in paraffin and cut into 5 μm -thick sections, followed by Fast Green FCF (0.02%) and Safranin O (0.1%) staining. The Osteoarthritis Research Society International (OARSI) scoring system was used to evaluate histopathological changes in the joints.

In situ hybridization assay. Mice were euthanized with 3% sodium pentobarbital before extraction of their spines, which were fixed by overnight immersion in 4% formaldehyde. The ipsilateral L3-L5 dorsal root ganglia (DRG) was embedded and cut into 16 μm -thick sections. A mouse-specific *miR-183* probe was purchased from Roche (USA). In situ hybridization (ISH) of *miR-183* was performed using digoxigenin-labelled riboprobes (Roche), according to the manufacturer's instructions. After the paraffin sections were deparaffinized and dehydrated, they were digested with proteinase K at 37 $^{\circ}\text{C}$ for 30 minutes. After pre-hybridization for one hour, probe-containing hybridization solution was added dropwise to

each section at 95 $^{\circ}\text{C}$ for five minutes and overnight at 37 $^{\circ}\text{C}$. After washing with hybridization solution, the sections were dripped with mouse anti-digoxigenin biotin-labelled antibody working solution, and incubated in a humid chamber at 37 $^{\circ}\text{C}$ for 45 minutes. Then, the sections were dripped with peroxidase streptavidin complex working solution, and incubated for 45 minutes at 37 $^{\circ}\text{C}$. After the 3, 3'-diaminobenzidine (DAB) colouration of the sections, the cytoplasm was observed under an optical microscope. The brown-yellow particles indicated a positive reaction, and the nucleus was blue.

Immunofluorescence assay. After the harvested spines were stored in 30% sucrose (Wt/Vol), individual ipsilateral L3-L5 DRG was embedded in optical coherence tomography (OCT) (Tissue-Tek, 4583; Sakura, USA) and frozen, and then cut into 12 μm sections. The sections were incubated with the primary antibodies anti-F4/80 (ab6640) and anti-doublecortin (ab77450; both Abcam, UK), followed by incubation with corresponding isotype-specific secondary antibodies and labelling with either AlexaFluor-488 or AlexaFluor-633 (Invitrogen, Thermo Fisher Scientific, USA). The sections were observed and photographed under a fluorescence microscope. Micrographic images were exported to Adobe Photoshop CS 5.1 (Adobe, USA) and adjusted for brightness and contrast to reflect the true colours. F4/80 positive cells

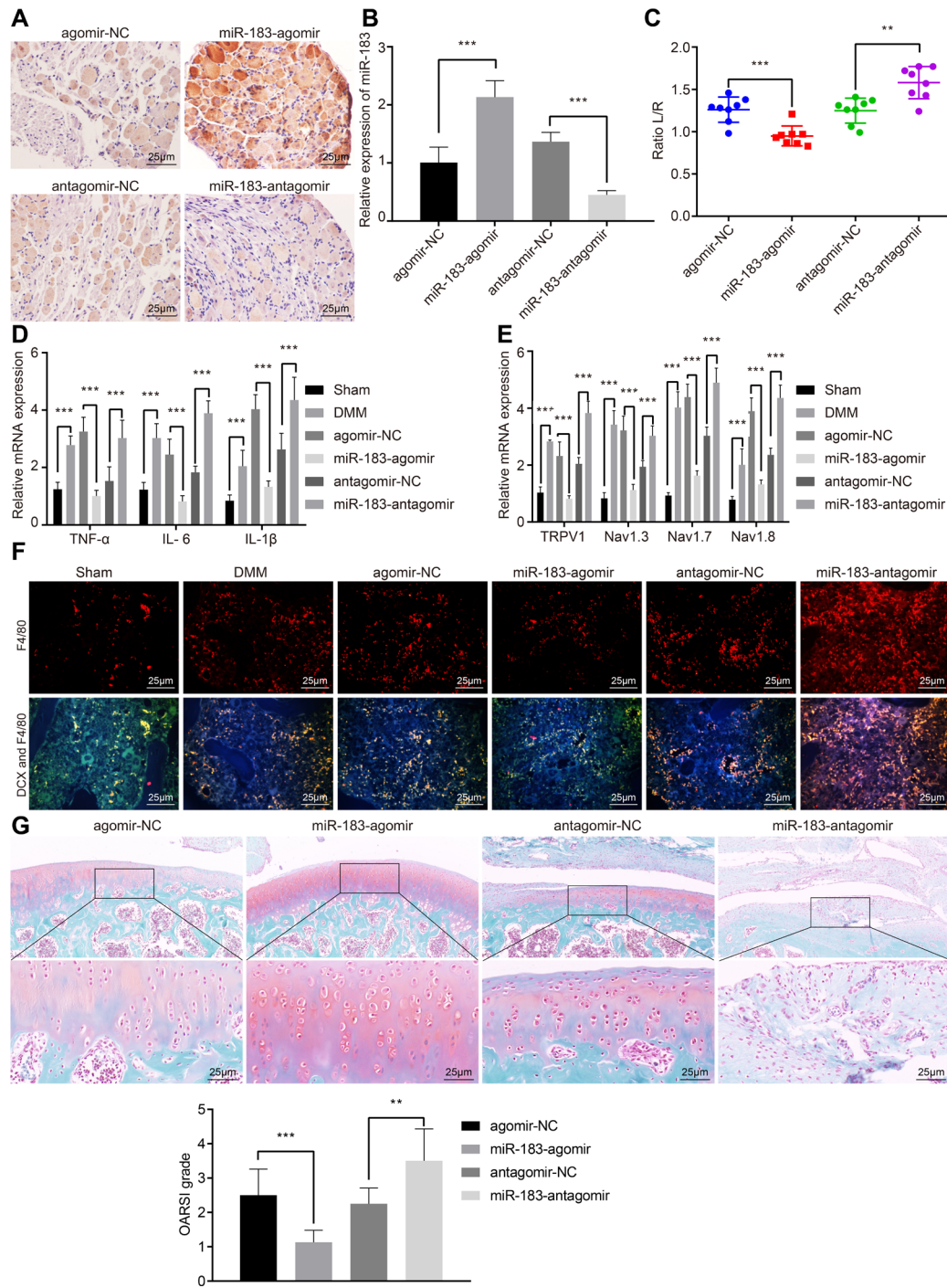


Fig. 2

Overexpression of microRNA-183 (miR-183) ameliorated osteoarthritis (OA) joint pain. a) In situ hybridization assay to evaluate miR-183 expression in mouse L3-L5 dorsal root ganglia (DRG) (400×). b) Quantitative reverse transcription polymerase chain reaction (RT-qPCR) evaluation of miR-183 expression in mouse L3-L5 DRG. c) Evaluation of mouse pain by behavioural assessment; the weight distribution between surgical and non-surgical side paws was measured using an IITS manometer ("Ratio L/R" refers to ratio of left and normal to right and arthritis). d) RT-qPCR evaluation of pro-inflammatory cytokine (tumour necrosis factor α (TNF-α), interleukin (IL)-6, IL-1β) levels in mouse L3-L5 DRG. e) Evaluation of pain-related factors (transient receptor potential vanilloid subtype-1 (TRPV1), voltage-gated sodium 1.3, 1.7, and 1.8 (Nav1.3, Nav1.7, and Nav1.8)) in mouse L3-L5 DRG. f) Immunofluorescent analysis of macrophage infiltration in mouse L3-L5 DRG (400×). g) Morphological analysis of mouse bone and joint tissue (400×) using Fast Green FCF (0.02%) and Safranin O (0.1%) staining, followed by Osteoarthritis Research Society International (OARSI) scoring. **p < 0.01; ***p < 0.001. The results were measurement data, and expressed as means (standard deviation (SD)). Comparisons among multiple groups were analyzed by analysis of variance (ANOVA), followed by Tukey's post hoc test. n = 8 mice in each group. Experiments were repeated three times. DCX, doublecortin; DMM, destabilization of the medial meniscus; mRNA, messenger RNA; NC, negative control.

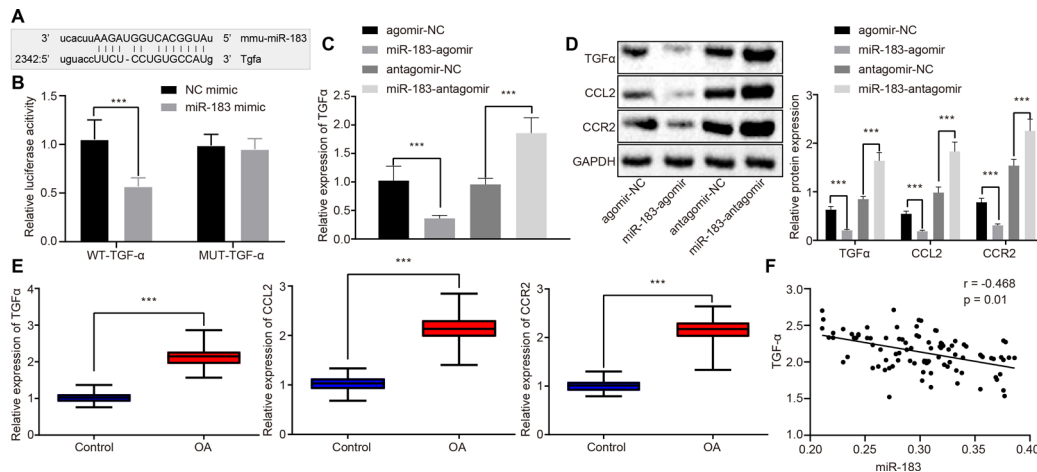


Fig. 3

Transforming growth factor α (TGF α) expression and C-C motif chemokine ligand 2 (CCL2)/C-C chemokine receptor type 2 (CCR2) signalling were inhibited by microRNA-183 (miR-183). a) Predicted miR-183 binding site in the TGF α three prime untranslated region (3'UTR). b) Dual luciferase reporter assay confirmed the direct binding of miR-183 and TGF α ; the experiment was repeated three times. c) TGF α expression in mouse dorsal root ganglia (DRG) evaluated by quantitative reverse transcription polymerase chain reaction (RT-qPCR); $n = 8$ mice in each group. d) Representative images and quantitative analysis of immunoblotting of TGF α , CCL2, and CCR2 levels in mouse DRG; $n = 8$ mice in each group. e) Relative TGF α , CCL2, and CCR2 messenger RNA (mRNA) levels measured by RT-qPCR in samples from patients with osteoarthritis (OA); $n = 95$ patients. f) Correlation analysis of TGF α and miR-183 expression in patients with OA; $n = 95$ patients. *** $p < 0.001$. The results were measurement data, and expressed as means (standard deviation (SD)). Comparisons between two unpaired groups were analyzed using the independent-samples t -test. Comparisons among multiple groups were analyzed by analysis of variance (ANOVA), followed by Tukey's post hoc test. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MUT, mutated; NC, negative control; WT, wild-type.

showed red fluorescence, doublecortin positive cells showed green fluorescence, and cells expressing both at the same time appeared yellow.

Dual luciferase reporter assay. Target genes of miR-183 were predicted using the bioinformatics tool, TargetScan.²¹ A synthetic WT TGF α three prime untranslated region (3'UTR) gene fragment was introduced into the pMIR-reporter (Beijing Huayueyang Biological Technology, China). Based on the WT template, mutated (MUT) TGF α 3'UTR was generated and inserted into the empty pMIR-reporter between the same cloning sites. The correctly sequenced luciferase reporter plasmids (WT and MUT, respectively) were cotransfected into HEK293T cells, with miR-183 mimic or NC mimic, respectively. After 48 hours of transfection, cells were collected and lysed, with the luciferase activity measured.

RNA isolation and quantitation. Total RNA was extracted from tissue samples and reverse transcribed. Primers (Supplementary Tables i and ii) were synthesized by Invitrogen. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 were used as internal reference genes. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) experiments were performed on an ABI 7500 quantitative PCR machine (Thermo Fisher Scientific) using a SYBR Premix Ex Taq (Tli RNaseH Plus) kit (RR820A; Takara Bio, Japan). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis. Proteins were isolated from cells using radioimmunoprecipitation assay buffer and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After transfer onto polyvinylidene fluoride membranes, samples were blocked

with Tris-buffered saline containing Tween 20 (TBST) and 5% bovine serum albumin. Thereafter, membranes were incubated with diluted primary antibodies, as follows: anti- β -actin (ab179467, 1:5,000; Abcam), anti-TGF α (ab92486, 1:500; Abcam), anti-CCL2 (#2029, 1:1,000; Cell Signaling Technology (CST), USA), or anti-CCR2 (ab203128, 1:1,000; Abcam), overnight at 4°C. Subsequently, horseradish peroxidase-conjugated secondary antibody (ab150077, 1:1,000; Abcam) was applied and the membrane was developed using chemiluminescence reagent. Protein levels were quantified using ImageJ software (National Institutes of Health (NIH), USA). Relative standard error (SE) was measured by dividing the densitometry value of the target protein by that of the respective loading control.

Statistical analysis. All data were analyzed using SPSS v21.0 software (IBM, USA). Data are expressed as means (SD). Comparisons between paired groups with normally distributed data and homogeneous variances were conducted by paired t -test. Comparison between unpaired groups with adjacent distribution and homogeneous variance was conducted using independent-samples t -test. Comparisons among multiple groups were conducted by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Comparisons among groups at different timepoints were conducted by repeated measures analysis of variance (ANOVA), followed by Bonferroni's post hoc test. A p -value < 0.001 was regarded as statistically significant.

Results

miR-183 downregulation in OA. RT-qPCR results showed that miR-183 expression was lower in tissue

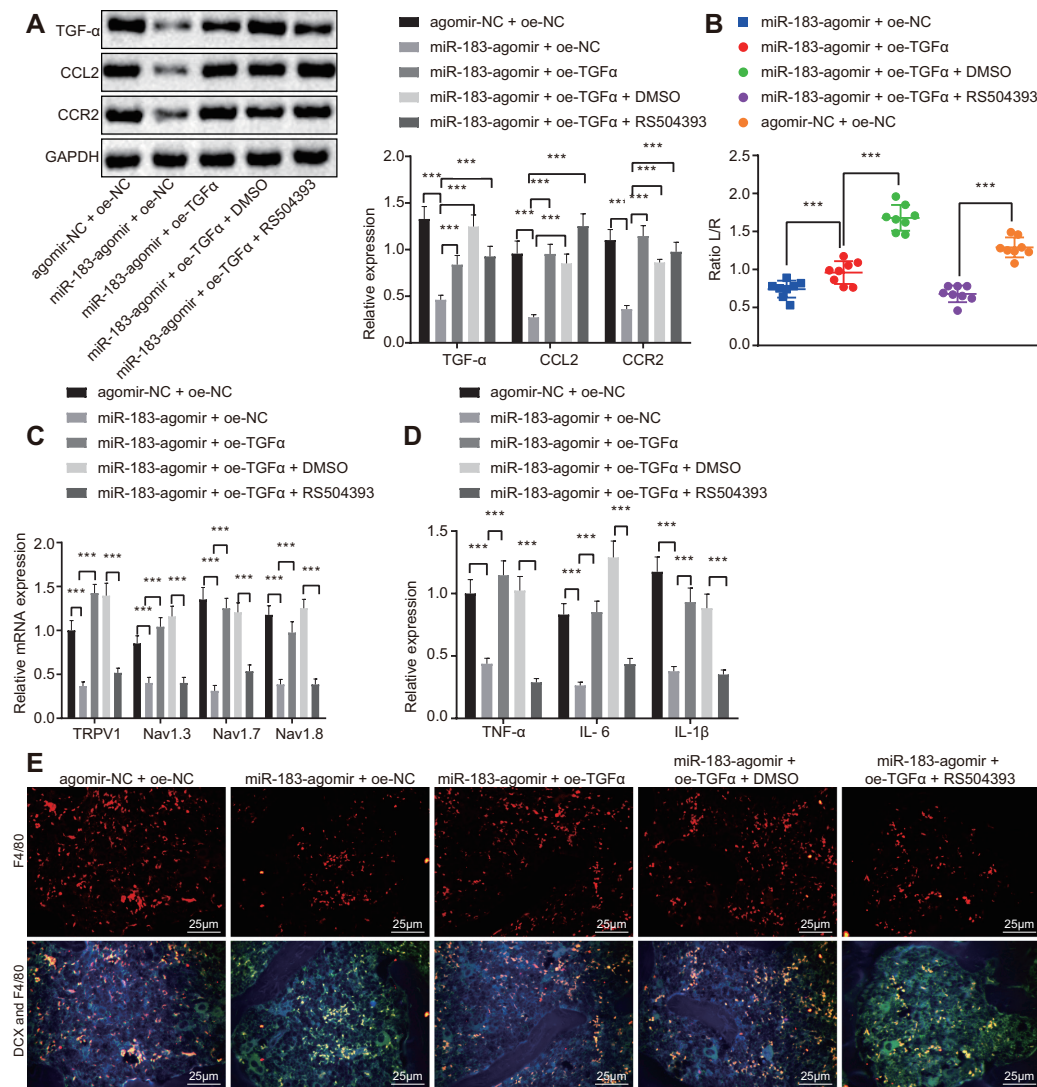


Fig. 4

microRNA-183 (miR-183) ameliorated osteoarthritis (OA) joint pain by inhibiting TGF α -CCL2/CCR2 signalling. a) Representative immunoblotting images and quantitative analysis of the expression of TGF α , CCL2, and CCR2. b) Evaluation of mouse pain by behavioural assessment; the weight distribution between surgical and non-surgical side paws was measured using an IITS manometer ("Ratio L/R" refers to ratio of left and normal to right arthritis). c) Expression of mouse L3-L5 dorsal root ganglia (DRG) pain-related factors examined by reverse transcription polymerase chain reaction (RT-qPCR). d) Expression of proinflammatory cytokines in L3-L5 DRG examined by RT-qPCR in mice. e) Representative images of immunofluorescence for macrophage infiltration in mouse L3-L5 DRG (400 \times). *** p < 0.001. The results were measurement data, and expressed as means (standard deviation (SD)). The independent-samples t -test was used for comparisons between the two groups. Comparisons among multiple groups were analyzed by analysis of variance (ANOVA), followed by Tukey's post hoc test. Data comparisons among groups at different timepoints were performed by repeated measures ANOVA. N = 8 mice in each group. Experiments were repeated three times. CCL2, C-C motif chemokine ligand 2; CCR2, C-C chemokine receptor type 2; DCX, doublecortin; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; Nav1.3, voltage-gated sodium 1.3; Nav1.7, voltage-gated sodium 1.7; Nav1.8, voltage-gated sodium 1.8; NC, negative control; oe, overexpressed; TGF α , transforming growth factor α ; TRPV1, transient receptor potential vanilloid subtype-1.

samples from patients with OA than that in those without OA (p < 0.001, independent-samples t -test, Figure 1a). Moreover, histomorphometric analysis (p < 0.001, independent-samples t -test, Figure 1b) revealed significantly reduced cartilage and increased joint pain (p < 0.001, independent-samples t -test, Figure 1c) in DMM mice (p < 0.001). Further, ISH and RT-qPCR assays showed that *miR-183* expression was reduced in DRG of DMM mice (p < 0.001, independent-samples t -test, Figures 1d and 1e). These results indicated that *miR-183* was poorly expressed in OA.

microRNA-183 ameliorated joint pain in OA. Next, we silenced or overexpressed *miR-183* in DMM mice, as confirmed by ISH and RT-qPCR (p < 0.001, one-way ANOVA, Figures 2a and 2b). Subsequently, a behavioural assay was performed to evaluate mouse pain, and the results showed that overexpression of *miR-183* diminished pain, while silencing of *miR-183* increased pain (p < 0.001, one-way ANOVA, Figure 2c).

RT-qPCR results (TNF- α : p < 0.001; IL-6: p < 0.001; IL-1 β : p < 0.001, Figures 2d and 2e; TRPV1: p < 0.001; Nav1.3: p < 0.001; Nav1.7: p < 0.001; Nav1.8: p < 0.001,

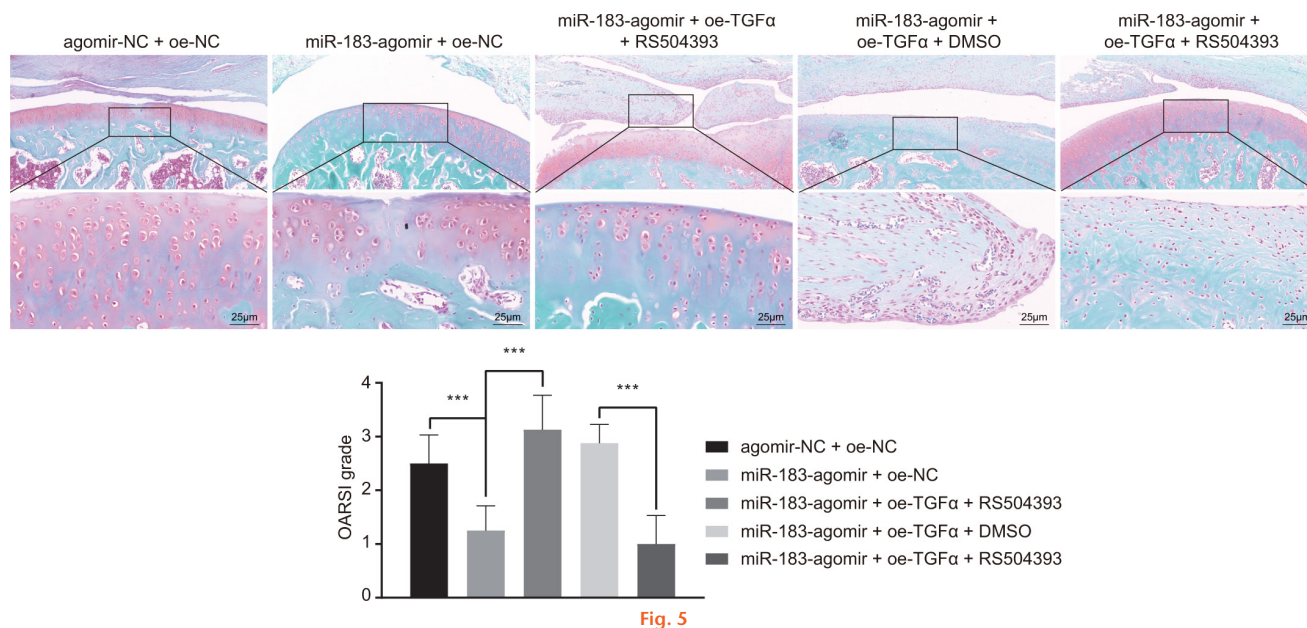


Fig. 5

microRNA-183 (miR-183) inhibited TGF α -CCL2/CCR2 signalling to alleviate the extent of osteoarthritis (OA). Morphological analysis of mouse bone and joint tissues (400 \times) using Fast Green FCF (0.02%) and Safranin O (0.1%) staining was conducted, followed by Osteoarthritis Research Society International (OARSI) scoring. *** $p < 0.001$. The results were measurement data, and expressed as means (standard deviation (SD)). Comparisons among multiple groups were analyzed by analysis of variance (ANOVA), followed by Tukey's post hoc test. $n = 8$ mice in each group. Experiments were repeated three times. DMSO, dimethyl sulfoxide; NC, negative control; oe, overexpressed; TGF α , transforming growth factor α .

all one-way ANOVA) showed that the inflammatory factors, IL-6, IL-1 β , and TNF- α , and the pain-related factors (transient receptor potential vanilloid subtype-1 (TRPV1), voltage-gated sodium 1.3, 1.7, and 1.8 (Nav1.3, Nav1.7, and Nav1.8)) were upregulated in DRG of DMM mice. Notably, this upregulation was inhibited in response to miR-183 overexpression and promoted by miR-183 silencing ($p < 0.05$). Consistent with these results, immunofluorescence staining showed that increased miR-183 resulted in decreased macrophage infiltration in DRG of DMM mice, whereas silencing of miR-183 led to an increase in macrophage infiltration (Figure 2f). Moreover, the OARSI scoring system and the quantitative results of articular cartilage histomorphology showed that elevated miR-183 reduced the extent of OA, while decreased miR-183 expression led to an increase in the extent of OA ($p < 0.001$, one-way ANOVA, Figure 2g).

miR-183 inhibited TGF α and CCL2/CCR2 signal. Bioinformatics analysis using TargetScan²¹ predicted that miR-183 binds to TGF α (Figure 3a). It has been documented previously that TGF α affects the occurrence of OA in a CCL2-dependent manner.¹⁵ Dual luciferase reporter assay (Figure 3b) showed that the luciferase activity of WT-TGF α was reduced in mice treated with miR-183 mimic relative to those treated with NC mimic ($p < 0.001$, independent-samples t -test), while the luciferase activity of MUT-TGF α showed no significant difference ($p = 0.505$, independent-samples t -test), indicating that miR-183 directly targets TGF α . Then, we further detected the expression of TGF α , CCL2, and CCR2 in mice subjected to different

treatments. We found that mice overexpressing miR-183-agomir consistently showed significantly reduced expression of TGF α (mRNA expression: $p < 0.001$, independent-samples t -test; protein expression: $p < 0.001$, independent-samples t -test), CCL2 ($p < 0.001$, independent-samples t -test), and CCR2 ($p < 0.001$, independent-samples t -test), while mice overexpressing miR-183-antagomir expressed increased levels of TGF α (mRNA expression: $p < 0.001$, $t = 15.57$; protein expression: $p < 0.001$, independent-samples t -test), CCL2 ($p < 0.001$, independent-samples t -test), and CCR2 ($p < 0.001$, independent-samples t -test) at both the messenger RNA (mRNA) and protein levels ($p < 0.05$) (Figures 3c and 3d). In agreement with these results in model mice, TGF α , CCL2, and CCR2 mRNA levels in samples from patients with OA were also upregulated (TGF α : $p < 0.001$; CCL2: $p < 0.001$; CCR2: $p < 0.001$, all independent-samples t -test, Figure 3e). Correlation analysis revealed a negative correlation between miR-183 and TGF α expression in OA (Figure 3f). These results indicated that miR-183 inhibited TGF α , thereby blocking CCL2/CCR2 signalling.

miR-183 ameliorated joint pain of OA by inhibiting TGF α -CCL2/CCR2 signalling. Western blot results (TGF α : $p < 0.001$; CCL2: $p < 0.001$; CCR2: $p < 0.001$, all one-way ANOVA, Figure 4a) showed that levels of TGF α , CCL2, and CCR2 were higher in mice injected with agomir-NC + oe-NC, relative to those injected with miR-183-agomir + oe-NC. Compared with mice injected with miR-183-agomir + oe-NC, levels of TGF α , CCL2, and CCR2 were increased in mice injected with miR-183-agomir + oe-TGF α ,

miR-183-agomir + oe-TGF α + DMSO, and miR-183-agomir + oe-TGF α + RS504393 (CCR2 agonist).

The pain behavioural assay results ($p < 0.001$, one-way ANOVA, Figure 4b) showed decreased pain in mice injected with miR-183-agomir + oe-NC relative to those injected with agomir-NC + oe-NC. The pain of the mice injected with miR-183-agomir + oe-TGF α was increased relative to that in mice injected with miR-183-agomir + oe-NC. The mice injected with miR-183-agomir + oe-TGF α + RS504393 (CCR2 agonist) exhibited significantly lower pain than those injected with miR-183-agomir + oe-TGF α + DMSO.

We also observed decreased proinflammatory cytokines (IL-6, IL-1 β , and TNF- α) and pain-related factors (TRPV1, Nav1.3, Nav1.7, and Nav1.8) in DRG from mice injected with miR-183-agomir + oe-NC compared with control littermates (mice injected with agomir-NC + oe-NC) ($p < 0.05$) (TRPV1: $p < 0.001$; Nav1.3: $p < 0.001$; Nav1.7: $p < 0.001$; Nav1.8: $p < 0.001$, Figures 4c and 4d; TNF- α : $p < 0.001$; IL-6: $p < 0.001$; IL-1 β : $p < 0.001$, all one-way ANOVA). In contrast, overexpression of TGF α neutralized the effect of miR-183 on mediating decreased proinflammatory cytokine levels and pain-related factors (Figures 4c and 4d), indicating that miR-183 downregulates those cytokines and pain-related factors in a TGF α -dependent manner. Interestingly, further blocking CCR2 using the CCR2 agonist, RS504393, also attenuated the effect of TGF α (Figures 4c and 4d). Taken together, these data indicate that miR-183 regulates TGF α , thereby exerting a regulatory effect on the CCL2/CCR2 axis.

Accumulating evidence supports the function of CCL2 as a chemoattractant important in macrophage regulation.²² Therefore, we evaluated macrophage infiltration in mouse DRG by immunofluorescent staining. In the miR-183-agomir + oe-NC group, infiltration was reduced compared with that in the agomir-NC + oe-NC group. Furthermore, macrophage infiltration in the miR-183-agomir + oe-TGF α + RS504393 (CCR2 agonist) group was significantly decreased relative to that in the miR-183-agomir + oe-TGF α + DMSO group (Figure 4e). These data further demonstrate that miR-183 attenuates OA joint pain by regulating the TGF α -CCL2/CCR2 signalling axis.

miR-183 alleviated the progression of OA by inhibiting TGF α -CCL2/CCR2 signalling. Evaluation using the OARSI scoring system and articular cartilage histomorphometric analysis demonstrated that the degree of inflammation and bone sclerosis in mice injected with miR-183-agomir + oe-NC was reduced compared with those injected with agomir-NC + oe-NC. Compared with the mice injected with miR-183-agomir + oe-NC, the degree of OA and osteosclerosis was exaggerated in those injected with miR-183-agomir + oe-TGF α ($p < 0.001$, one-way ANOVA). Moreover, this effect was reversed by additional injection of the CCR2 agonist, RS504393 (Figure 5). In summary, miR-183 can alleviate OA progression by inhibiting the TGF α -CCL2/CCR2 signalling axis.

Discussion

OA is the most frequently occurring inflammatory joint disease, and causes pain and impaired mobility in people across the globe,²³ affecting approximately 240 million individuals;²⁴ however, early diagnosis of OA is not currently feasible,²⁵ and available treatments for OA are not fully effective and usually have side effects.²⁶ Pain is the major symptom of OA, and NSAIDs are the most common treatment option; however, NSAIDs cannot completely relieve the pain of OA and long-term use of NSAIDs is associated with health risks.²⁷ The origin and mechanisms underlying OA joint pain remain unclear.²⁸ In this study, we found that miR-183 could ameliorate OA pain through inhibiting the TGF α -CCL2/CCR2 signalling axis.

Our initial results showed that miR-183 expression was reduced in joint tissue from patients with OA and murine OA models. Furthermore, miR-183 overexpression inhibited the production of proinflammatory cytokines, such as IL-6, IL-1 β , and TNF α . Simultaneously, pain-related factors, including TRPV1, Nav1.3, Nav1.7, and Nav1.8, were also downregulated. IL-6 has a vital role in mediating inflammation and consequent metabolic changes,²⁹ while IL-1 β is a pro-inflammatory factor and a therapeutic target in some chronic autoimmune conditions.³⁰ In contrast, TGF α and TGF- β 1 are mainly known as anti-inflammatory factors.³¹ Transient receptor potential ankyrin 1 (TRPA1) is generally considered to be a pain receptor in sensory neurones, and inhibition of TRPV1 is reported to participate in the inhibitory effect of the TRPA1 ion channel on T cell-mediated colitis.³² Concerning pain-related factors, increased expression of certain Nav isoforms in peripheral sensory neurones is related to chronic and neuropathic pain conditions.³³ Consistent with our findings, targeting the miR-183 cluster can treat OA and alleviate joint pain.¹² The miR-183 cluster can regulate basal and neuropathic pain genes to rescale mechanical pain sensitivity.³⁴ Macrophages are critical players in the innate immune system, and constitute the first line of defence against pathogen invasion and in regulating adaptive immunity.³⁵ Moreover, macrophages are important in OA pathology.³⁶ In this study, we found that miR-183 inhibited macrophage infiltration in affected joints. OA is caused by destruction of osteoclast formation and function.³⁷ The results of this study revealed that miR-183 ameliorated OA.

Further, we found that miR-183 overexpression inhibited TGF α expression, thereby downregulating CCL2/CCR2 signalling. Consistent with our findings, TGF α levels were increased in OA cartilage from rats and humans.¹⁵ Furthermore, CCL2 and CCR2 are crucial to the development of pain associated with knee OA.³⁸ CCL2 expression is higher in articular cartilage samples from patients with OA compared with controls,³⁹ and CCR2+ cells are abundant in human OA synovium.²⁰ TGF α is documented as a major factor involved in inducing OA pain,⁴⁰ while CCL2/CCR2 signalling is an important trigger in the progression of pain in murine OA.⁴¹ TGF α induces

cartilage degradation by a CCL2-dependent mechanism in experimental post-traumatic OA.¹⁵ In this experiment, TGF α , CCL2, and CCR2 expression levels were inhibited by miR-183 overexpression, and together alleviated OA pain. Further, through inhibiting the expression of TGF α , CCL2, and CCR2, miR-183 ameliorated the extent of OA.

In conclusion, based on these findings, we propose that miR-183 inhibits the expression of TGF α and consequently suppresses CCL2/CCR2 signalling to alleviate OA pain (Supplementary Figure a), thus providing a promising therapeutic target for OA. However, additional works should be performed to warrant further exploration in the clinical setting.

Supplementary material



Figure depicting the mechanism of this study. Tables showing primer sequences for human quantitative reverse transcription polymerase chain reaction (RT-qPCR) and mouse primer sequences for RT-qPCR. ARRIVE checklist also included.

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Funding statement:

- This study was supported by the Key research and development projects of Hunan Province (No. 2020SK2075). No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

Open access funding:

- Open access funding was provided by the Key research and development projects of Hunan Province (No. 2020SK2075).

Acknowledgements:

- Not applicable.

Ethical review statement:

- This study was approved by the Ethics Committee and Animal Care and Use committee of our hospital. All participating patients signed informed consent documents. The animal experiments were performed in compliance with the National Institute of Health's Guidelines of Laboratory Animal Care and Use in Biomedical Research, and the ARRIVE checklist was completed to show that ARRIVE guidelines were adhered to in this study.

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