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ORIGINAL ARTICLE

Intra-Articular Injection of miR-29a-3p of BMSCs Promotes Cartilage Self-Repairing and Alleviates Pain in the Rat Osteoarthritis

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Abstract

BACKGROUND: Stem cells intra-articular injection stagey indicated a potential therapeutic effect on improving the pathological progress of osteoarthritis (OA). However, the long-term effect of stem cells intra-articular injection on the cartilage regeneration remains unclear. miR-29a-3p is predicted to be a critical target for inhibiting insulin-like growth factor-1 expression and may aggravate the progression of OA.

METHODS: In this study, we investigated the therapeutic efficacy of intra-articular injection of bone marrow mesenchymal stem cells (BMSCs) transfected with miR-29a-3p inhibitor in OA.

RESULTS: miR-29a-3p inhibitor transfection did not influence cell viability of BMSCs, while the chondrogenic differentiation potential of BMSCs was significantly improved. Interestingly, intra-articular injection of BMSCs with miR-29a-3p inhibition significantly prevented articular cartilage degeneration by up-regulating the expression of *Sox 9, Col-2a1, aggrecan* and down-regulating the expression of matrix metalloproteinase, as well as relieved pain in OA.

CONCLUSION: The double effects on cartilage protection and pain relief indicated a great potential of intra-articular injection of miR-29a-3p inhibitor-transfected BMSCs for the treatment of OA.

Keywords Osteoarthritis · BMSCs · miR-29a-3p · Cartilage · Pain

1 Introduction

Osteoarthritis (OA) is a chronic bone and joint disease, characterized by degeneration and disappearance of cartilage, reactive hyperplasia of ligaments and subchondral bone at the joint edge, resulting in joint pain, stiffness and dysfunction. Due to the limited ability of articular cartilage regeneration in adults, articular cartilage degeneration is considered as an irreversible pathological change. According to statistics, about 80% of the people over 65 years old in China suffer from OA, which is one of the main reasons for the physical disability of the elderly. Finally, arthroplasty has to be taken to treat OA, but it often brings heavy economic burden, long recovery time, and catastrophic complications such as infection, prosthesis displacement, and refracture. Therefore, it is of great significance to develop early non-surgical treatment of OA. However, the existing conservative treatment strategies, such as taking non-steroidal anti-inflammatory drugs, intra-articular injection of glucocorticoid, hyaluronic acid, etc., have not achieved ideal outcomes, and can only temporarily alleviate the disease progression.

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With the rapid development of bone tissue engineering and cartilage regenerative medicine, the use of stem cells and cytokines has gradually become a new strategy for the treatment of OA. Among them, mesenchymal stem cells (MSC), especially bone marrow mesenchymal stem cells (BMSCs), have become important seed cells for the treatment of OA due to their advantages of obtaining easily and extensive sources, advanced proliferation ability, and significant chondrogenic differentiation potential. The interest in stem cells was aroused from a study using intra-articular injection of BMSCs in OA goat model, reported by Murphy et al. [1]. Since then, series of clinical researches have been conducted by a similar technique and have shown promising applications [2–4]. As we all know, MSCs have the potential of multi-directional differentiation and sustain the ability to regulate inflammation, which has been widely used to repair cartilage and ameliorate the development of osteoarthritis[5–7]. However, after intra-articular injection, if there are only a few injected cells adhering to the cartilage defect, the differentiation ability of MSCs is not prominent [8]. In addition, the chondrogenic differentiation of MSCs after intra-articular injection is not well controlled, which may limit their potential in clinical applications [9, 10]. Therefore, we believe that MSC with more chondrogenic differentiation ability will contribute to the efficient repair of articular cartilage in OA.

Regardless of cell source, MSC can differentiate into chondrocytes under the stimulation of inducers including transforming growth factor-β (TGF-β), bone morphogenetic protein-7 (BMP-7), insulin-like growth factor-1 (IGF-1), and so on [11]. Among them, IGF-1 is one of the most important growth factors in the regulation of cartilage development and cartilage homeostasis. It can stimulate the proliferation of chondrocytes and promote the synthesis of type II collagen (Col-2) and proteoglycan to maintain the phenotype of chondrocytes. Therefore, IGF-1 may be an effective target for the treatment of cartilage defects. Therefore, MSC edited by IGF-1 may provide a new strategy for the treatment of OA.

MicroRNA (miRNA) is group of highly conserved endogenous, small non-coding RNA, and it can modulate gene expression by directly inhibiting targets at the transcriptional level or prohibiting proteins translation at the post-transcriptional level [12]. Recently, miRNAs showed high efficiency to modulate stem cells fate determination and tissue regeneration [13, 14]. Emerging evidence presented the interesting role of some miRNAs in chondrogenesis and OA occurrence [15]. Predicted by Target.Scan, miR-29a-3p may target the 3'-untranslated region (3'-UTR) of IGF-1. Therefore, miR-29a-3p regulating IGF-1 in MSCs may affect the cell functions of MSCs, and then influence its differentiation.

In this study, the regulation of miR-29a-3p knockdown on the expression of IGF-1 and chondrogenic differentiation markers were evaluated in BMSCs *in vitro*. Then, the effects of intra-articular injection of BMSCs with miR-29a-3p inhibition were confirmed by detecting the BMSCs differentiation ability, articular cartilages morphology, cartilage degeneration and pain relieving potential in OA *in vivo*. The study aimed to enhance the potential application of BMSCs in treating OA by miRNA targeting and provide a novel approach to ameliorate OA.

2 Materials and methods

2.1 Materials

Low Glucose Dulbecco's Modified Eagle's Medium (LG-DMEM), streptomycin-penicillin and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). Chondro-inductive medium was supplied by Cyagen (Guangzhou, China) and TGF-β3 was obtained from PeproTech (Cranbury, NJ, USA). Sprague–Dawley rats BMSCs RASMX-01001 was obtained from Cyagen Biosciences (Guangzhou, China). Cell Counting Kit-8 (CCK-8) C0039 and Calcein acetoxymethyl ester (Calcein-AM)/Propidium Iodide (PI) C2015M were purchased from Beyotime Institute of Biotechnology (Shanghai, China). The 4% paraformaldehyde solution, phosphate buffer saline (PBS), and Triton X-100 were obtained from Beijing Solarbio Science & Technology (Beijing, China). Eastep Super Total RNA Extraction Kit was supplied by Promega Corporation. Perfect Real Time RT reagent kit, Prime Script RT reagent kit, and SYBR Premix Ex Taq II kit were provided by Takara Biotechnology (Dalian, China). TRIzol Reagent was supplied by Invitrogen Life Technology (Carlsbad, CA, USA). Safranine O, alcian blue, hematoxylin eosin (H&E), and safranin O and fast green stains were obtained from Thermo Fisher Scientific (Shanghai, China). Antibodies used for immunofluorescence were purchased from Abcam (Cambridge, UK).

2.2 Cell culture and miR-29a-3p inhibitor lentivirus transfection

BMSCs were maintained in LG-DMEM containing 10% FBS and 1% streptomycin-penicillin. Cultures of BMSCs were maintained at 37 °C in a humidified atmosphere of 5% CO_2. miR-29a-3p inhibitor lentivirus were provided by GenePharma (Shanghai, China). The sequence of miR-29a-3p inhibitor was 5'-UUCGUGGUUCACUUUACGGAAA-3'. BMSCs in the logarithmic growth phase were seeded in 6-well plates. After reaching 70%-80% confluence, cells were transfected with 50 nmol miR-29a-3p inhibitor

lentivirus or negative control vector. Five hour after transfection, fresh medium was replaced. The specific grouping and abbreviation of *in vitro* cell experiment are as follows: BMSCs without transfection was abbreviated as NC group, BMSCs transfected with vector was abbreviated as LV-NC group, and BMSCs transfected with miR-29a-3p inhibitor lentivirus was abbreviated as LV-miR-29a-3p inhibitor group.

2.3 Real-time quantitative PCR (RT-qPCR)

To detect the expression of *IGF-1* in transfected cells, and the gene expression of *Sox 9*, *Col-2a1*, *aggrecan*, and *matrix metalloproteinase (MMP)*-13 in the cells and/or cartilage tissue, RT-qPCR was carried out. Chondro-inductive medium containing 10 ng/mL TGF-β3 was used to chondrogenic induction *in vitro*. The Chondro-inductive medium was changed every three days. Briefly, total RNA was extracted with TRIzol reagent, and cDNA was synthesized in reverse transcription reaction by 1 ug total RNA using a Prime Script RT reagent kit according to the manufacturer's protocols. The expression of target genes was detected by RT-qPCR using the SYBR Premix Ex Taq II kit according to the manufacturer's protocol. Amplification was performed in 96-well optical reaction plates on the LightCycler 480 (Roche Diagnostics, Basel, Switzerland). *GAPDH* and *U6* were used as internal controls for mRNA and miRNA expression, respectively. Primer sequences of genes were displayed in Table 1. The relative RNA expression were calculated by the formula of $2^{-\Delta\Delta Ct}$ method [16].

2.4 Western blotting

To study the expression of IGF-1 in BMSCs transfected with miR-29a-3p inhibitor at the translational level, we cultured the transfected BMSCs for 3 days and detected the protein expression by western blotting. Briefly, the total protein was collected from BMSCs with different treatment. After the lysates were centrifuged, protein concentrations were determined. And then the supernatant proteins were collected, denatured, separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) with 5% stacking gels and 10% separating gels, and transferred. The membranes were incubated with primary antibody overnight at 4 °C after blocking with 5% BSA at room temperature for 1 h. Membranes were then incubated with secondary antibodies for 40 min. The grey bands were calculated using Image J software.

Table 1 Primer sequences of genes

Gene	Oligonucleotide Primers (5′-3′)
Sox 9	F: 5′- AGC GAA CGC ACA TCA AGA C-3′
	R: 5′- CTG TAG GCG ATC TGT TGG GG-3′
Col-2a1	F: 5′- TGG ACG ATC AGG CGA AAC C-3′
	R: 5′- GCT GCG GAT GCT CTC AAT CT-3′
Aggrecan	F: 5′- AGA ATC CAC CAC CAC CAG-3′
	R: 5′- ATG CTG GTG CTG ATG ACA-3′
MMP-13	F: 5′-CTC ACA GAC CTG ACT CGG TT-3′
	F: 5′-CAC GCC TGA AGG AAG AGA TG-3′
GAPDH	F: 5′- GGA GCG AGA TCC CTC CAA AAT-3′
	R: 5′- GGC TGT TGT CAT ACT TCT CATGG-3′

2.5 Luciferase assay

Luciferase constructs were established by ligating oligonucleotides containing the wild-type (WT) or mutant (MUT) putative target site of the IGF-1 3′-UTR into the multiple cloning site of the pmirGLO vector (Promega, Madison, WI, USA). Constructs were verified by sequencing. Human embryonic kidney 293 (HEK293) cells were cultured in 24-well plates at 2.5×10^5 cells/well in DMEM/F-12 (Hyclone) supplemented with 10% FBS (Gibco) without antibiotics for 24 h. HEK293 cells were co-transfected with WT constructs, MUT constructs or vector and miR-29a-3p mimic or scramble miRNA using Lipofectamine 2000 (Invitrogen). The sequence of miR-29a-3p mimic was 5′-UAGCACCAUCUGAAAUCGGUUA-3′, and scramble miRNA was 5′-UCAUCAAGUCUGAAAUCGGUUA-3′. The sequence of WT 3′-UTR of IGF-1 was 5′-CCAGCUAUGCCAAUGUGGUGCUA-3′, and MUT 3′-UTR was 5′-CCAGCUAUGCCAAUGCAACAUCG-3′. After transfecting for 5 h, the medium was replaced with fresh medium containing 10% FBS. After 48 h, luciferase activity was detected by the Dual-Glo Luciferase Assay System (E2920; Promega), via the manufacturer's protocol. Light emission was detected by a GloMax 96 Microplate Luminometer (Promega). Firefly luciferase activity was normalized to that of Renilla luciferase for each sample.

2.6 Cells staining

BMSCs were seeded in 12-well plates at a density of 5×10^4 cells/well, and induced by TGF-β3-containing chondrogenic medium for 7 days, and then stained with safranin O and alcian blue. Briefly, the cells were fixed in 4% paraformaldehyde for 5 min and then washed with PBS. Subsequently, the samples were stained with safranin O for 5 min, and stained with alcian blue for 30 min at room temperature according to the manufacturer's instructions.

2.7 Cell viability assays

In order to detect the viability of BMSCs after transfection, CCK-8 assay and Calcein-AM/PI staining were performed. In brief, cells were seeded in 12-well plates at a density of 5×10^4 cells/well and incubated at 37 °C and in a 5% CO_2 atmosphere. After 1, 4 and 7 days of culture, CCK-8 assay was carried out for cell proliferation detection. The absorbance was quantitatively detected by a microplate reader (Multiskan EX; Thermo Fisher Scientific, Inc.) at 450 nm. In addition, to observe the cell survival, the cells were stained with the Calcein-AM/PI Double Stain kit according to the manufacturer's protocol, and observed under a FV1000 confocal laser scanning microscope (CLSM) (Olympus, Tokyo, Japan) after 3 days of culture. The cell survival rate was quantitatively analyzed using Image J 1.50i (NIH, Bethesda, MD, USA) according to the fluorescent images.

2.8 Rat OA model establishment and intra-articular injection

Sprague–Dawley rats (male, 8-week-old) were purchased from the Experimental Animal Center of Nanjing University of Chinese Medicine. All animal procedures were performed in accordance with the guidelines for Care and Use of Laboratory Animal Experience and approved by the Animal Care and Use Ethics Committee of Nanjing University of Chinese Medicine (Approval no. ACU191101). The rats were anesthetized by intraperitoneal injection of 3% pentobarbital at the dose of 0.2 ml/100 g, and the surgical destabilization of the medial meniscus (DMM) model of OA was prepared by medial collateral ligament transaction as described previously [17, 18].

Four weeks after DMM operation, the rats were divided into 4 groups (n = 10) randomly, and were intraarticularly injected with the following therapeutic agents, namely, PBS (abbreviated as NC group), 1×10^7 BMSCs in 100 µl PBS (abbreviated as BMSC group), 1×10^7 BMSCs transfected with vector in 100 µl PBS (abbreviated as BMSC-LV-NC group), and 1×10^7 BMSCs transfected with miR-29a-3p inhibitor lentivirus (abbreviated as BMSC-LV-miR-29a-3p inhibitor group).

At 6 and 12 weeks after intra-articular injection, the rats were sacrificed by overdose of pentobarbital and the articular cartilage was collected for RT-qPCR and histological examination.

2.9 Histological and immunohistochemical evaluation

After scarification, the articular cartilage of knee joints was collected and fixed in 4% paraformaldehyde. The joint samples were decalcified with 0.5 M EDTA solution for about 4 weeks and embedded for paraffin-sectioning. Then, 5 µm thick slices were prepared and stained with H&E and safranin O and fast green according to the standard protocols. The Osteoarthritis Research Society International (OARSI) cartilage histopathology assessment system and the Mankin scoring system were used to evaluate the destruction of knee cartilage [19]. In addition, the sections were incubated with primary antibodies against *Sox 9, Col-2a1, aggrecan, and MMP-13* for immunofluorescence staining in the cartilage.

2.9.1 Behavioral studies of the pain relief

To investigate the pain related behavioral changes in OA rats after intra-articular injection of therapeutic agents, paw withdrawal threshold (PWT) was measured to detect the effects on pain threshold as previous studied [20]. Briefly, the mechanical allodynia (hypersensitivity), PWT, was detected according to the force exerted by von Frey filament (Ugo Basile, Varese, Italy) ranging from 0 to 40 g with a 0.2 g accuracy. The paw sensitivity threshold was regarded as the minimum force for leading to a strong and immediate withdrawal reflex of the paw.

2.9.2 Statistical analyses

All results were calculated as mean ± standard deviation (SD) from at least 3 independent experiments. Comparisons among groups were analyzed with one-way ANOVA followed by Tukey's post hoc test using SPSS 19.0 (SPSS Inc.). $p < 0.05$ was considered as significant difference between groups. All histological images were analyzed by Image J 1.50i (NIH).

3 Results and discussion

3.1 Characterization of miR-29a-3p inhibitor-transfected BMSCs

Predicted by Target. Scan, there are two binding sites of miR-29a-3p on 3′-UTR on IGF-1 (Fig. 1A). To evaluate the regulatory role of miR-29a-3p in IGF-1, the expression levels of miR-29a-3p and IGF-1 were explored. As shown in Fig. 1B, after 48 h of transfection miR-29a-3p inhibitor, the level of miR-29a-3p expression in BMSCs was significantly inhibited than that in NC group. While, the

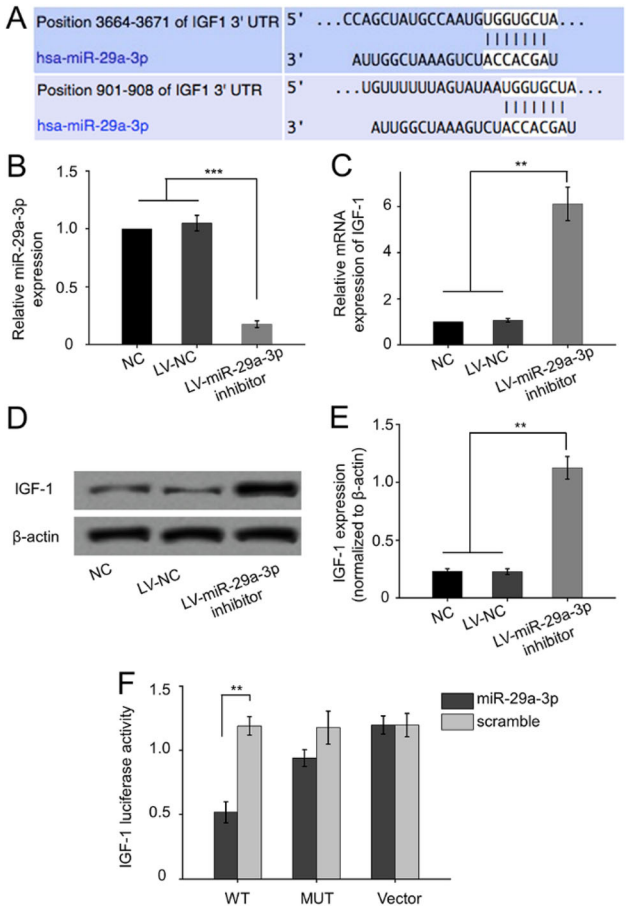

Fig. 1 The transfection of miR-29a-3p inhibitor significantly inhibited miR-29a-3p and enhanced IGF-1 in BMSCs. **A** Predicted binding sites of miR-29a-3p on 3′-UTR of IGF-1. **B** Relative miR-29a-3p expression in BMSCs after transfection of miR-29a-3p inhibitor. **C** Relative gene expression of *IGF-1* in BMSCs transfected with miR-29a-3p inhibitor. **D–E** Protein expression level of IGF-1 in BMSCs when transfected with miR-29a-3p inhibitor detected by western blot. **F** Luciferase activity with various reporters was detected in the presence or absence of miR-29a-3p mimic in HEK293 cells. $^{**}p < 0.01$, $^{***}p < 0.001$, $n = 6$

lentivirus vector transfection (LV-NC group) did not induce any significant change compared to NC group. Then, in order to identify the expression of IGF-1 in BMSCs transfected with miR-29a-3p inhibitor, RT-qPCR and Western blotting were conducted. The results demonstrated that expression of *IGF-1* gene was significantly increased in the cells with low expression of miR-29a-3p (Fig. 1C). Similarly, at the protein level, the expression of IGF-1 in LV-miR-29a-3p inhibitor group was significantly higher than NC group and LV-NC group (Fig. 1D–E). Lentivirus vector transfection did not affect the expression of IGF-1 in BMSCs. These results confirmed the knockdown of miR-29a-3p significantly up-regulated IGF-1 expression in BMSCs. Furthermore, luciferase assay using the WT or MUT version of the predicted binding site in the IGF-1 3′-UTR (Fig. 1F) revealed that cotransfection of HEK293 with the corresponding 3′-UTR WT plasmid and miR-29a-3p mimic resulted in a significant decrease in luciferase activity compared with the scrambled mimic. In contrast, miR-29a-3p mimic did not suppress the luciferase activity of the MUT plasmid or vector. This confirmed the direct inhibiting effect of miR-29a-3p on IGF-1. The above observations indicated that miR-29a-3p could directly regulate IGF-1 in BMSCs.

3.2 Cell viability of BMSCs was not affected by transfection of miR-29a-3p inhibitor

In addition to regulating the expression of target genes, a stem cell editing technology expected to achieve clinical effects needs to be harmless to the viability of stem cells. Therefore, the cell viability of BMSCs with different treatments was evaluated by the CCK-8 assay. As exhibited in Fig. 2A, with the time extension of the incubation, the absorbance of each group increased significantly. Moreover, the transfection did not inhibit the proliferation of BMSCs including LV-miR-29a-3p inhibitor group or negative control group (LV-NC group) at 1, 4, and 7 days, compared to NC group. These results revealed that the transfection conduction would not induce the cell toxicity to BMSCs.

Subsequently, Calcein-AM/PI staining was conducted to detect the cell survival (Fig. 2B). The fluorescence images demonstrated that the BMSCs in all group were almost presented with green-stained live cells, and the red-stained dead cells were limited. Through quantitative analysis, the survival rates in the NC group, LV-NC group, and LV-miR-29a-3p inhibitor group were $95.55 \pm 2.18\%$, $94.15 \pm 1.30\%$, and $94.77 \pm 1.93\%$, respectively. There was no distinct difference between these groups (Fig. 2C). These results clearly indicated that transfection of miR-29a-3p inhibitor lentivirus did not have side effect on cell viability of BMSCs. This kind of stem cell editing technology, which does not inhibit the cell viability, can efficiently regulate the expression of target genes and has the potential to be implicated in the therapeutic strategy in OA.

3.3 Knockdown of miR-29a-3p induces chondrogenic differentiation of BMSCs

To detect the function of miR-29a-3p inhibitor on the chondrogenic differentiation of BMSCs, the chondrogenic markers were explored by RT-qPCR. The results indicated that the expression of chondrogenic genes *Sox 9*, *Col-2a1*, and *aggrecan* were up-regulated in LV-miR-29a-3p inhibitor group, which were significantly higher than that of NC group and LV-NC group (Fig. 3A–C , $p < 0.05$). Moreover, safranine O and alcain blue staining were

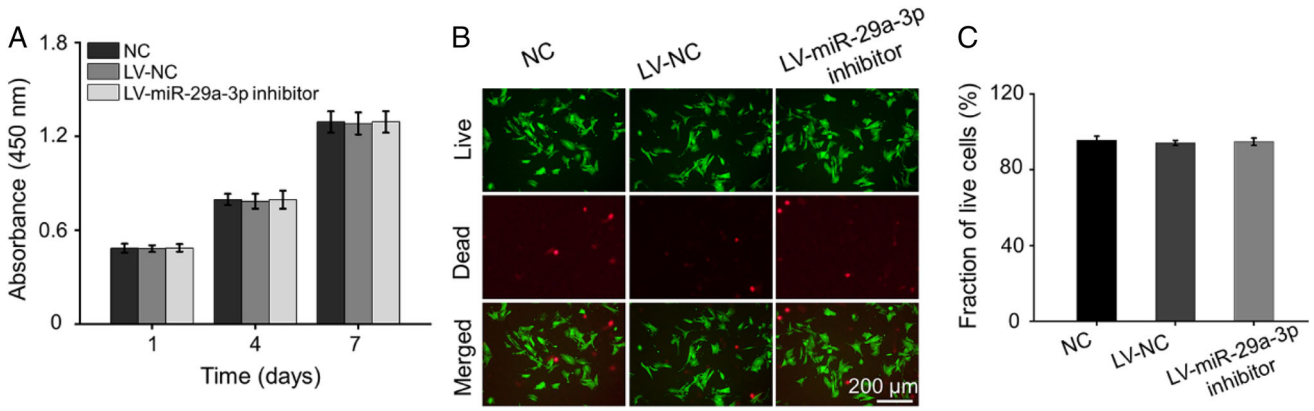

Fig. 2 Cell viability of BMSCs transfected with miR-29a-3p inhibitor. **A** The cell proliferation assay was studied with chondrocytes for 1, 4, and 7 days by CCK-8 assay. **B** Representative fluorescence microscopy images after Calcein-AM/PI staining of chondrocytes incubated for 3 days. Green fluorescence represented live cells, and red fluorescence indicated dead cells. **C** THE survival rates of BMSCs under different conditions. n = 6

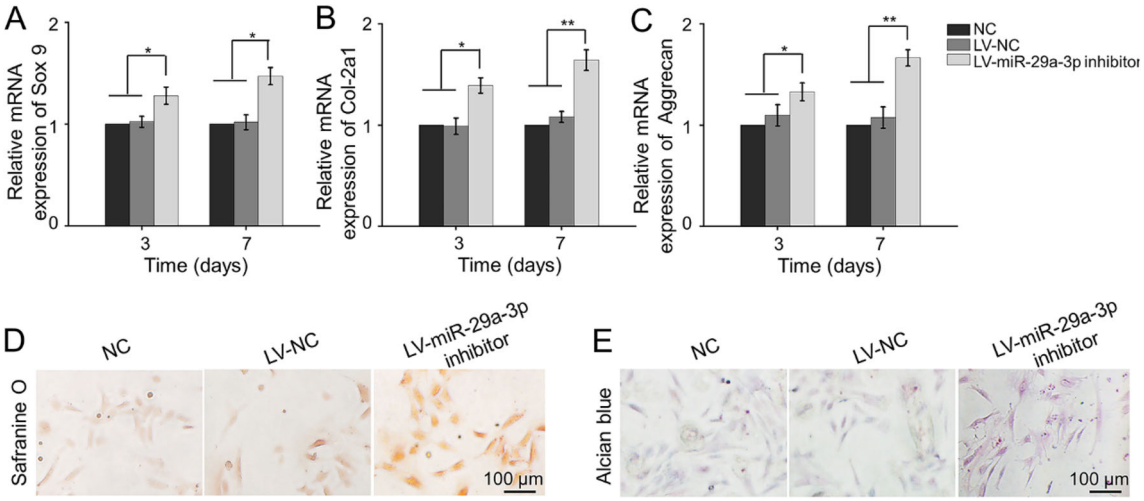

Fig. 3 Chondrogenic differentiation of BMSCs was stimulated with downregulation of miR-29a-3p. **A–C** RT-qPCR analysis of chondrogenic genes *Sox 9*, *Col-2a1*, and *aggrecan* of BMSCs transfected with miR-29a-3p inhibitor lentivirus. (**D–E**) Safranine O and alcain blue of BMSCs transfected with miR-29a-3p inhibitor lentivirus. $*p < 0.05$, $**p < 0.01$, n = 6

carried out to confirm the chondrogenesis of BMSCs transfected with miR-29a-3p inhibitor for 14 days. As shown in Fig. 3D–E, downregulation of miR-29a-3p could effectively promote chondrogenic differentiation of BMSCs with the enhanced expression. According to the above results, it can be revealed that miR-29a-3p inhibitor served as a stimulator of chondrogenic differentiation of BMSCs.

Chondrogenic differentiation of bone marrow mesenchymal stem cells *in vitro* mainly depends on the addition of induction factors. Previous studies have shown that TGF-β and IGF-1 are the main inducing factors [21–23]. TGF-β is a critical factor in the process of directing differentiation of BMSCs into chondrocytes. TGF-β can deliver a signal, which is transmitted to the nucleus through Smad pathway, to activate the expression of *Sox 9*, and then promote the expression of Col-2 [24]. However, TGF-β induction also has certain disadvantages, such as easy to lead to chondrocyte hypertrophy [25]. IGF-1 can induce BMSCs to differentiate into chondrocytes by promoting cell mitosis, cartilage matrix synthesis, and increasing Col-2 expression. Moreover, the side effects of IGF-1 inducing BMSCs are limited. However, there were only a few studies focusing on the regulation of *Sox 9, Col-2a1,*

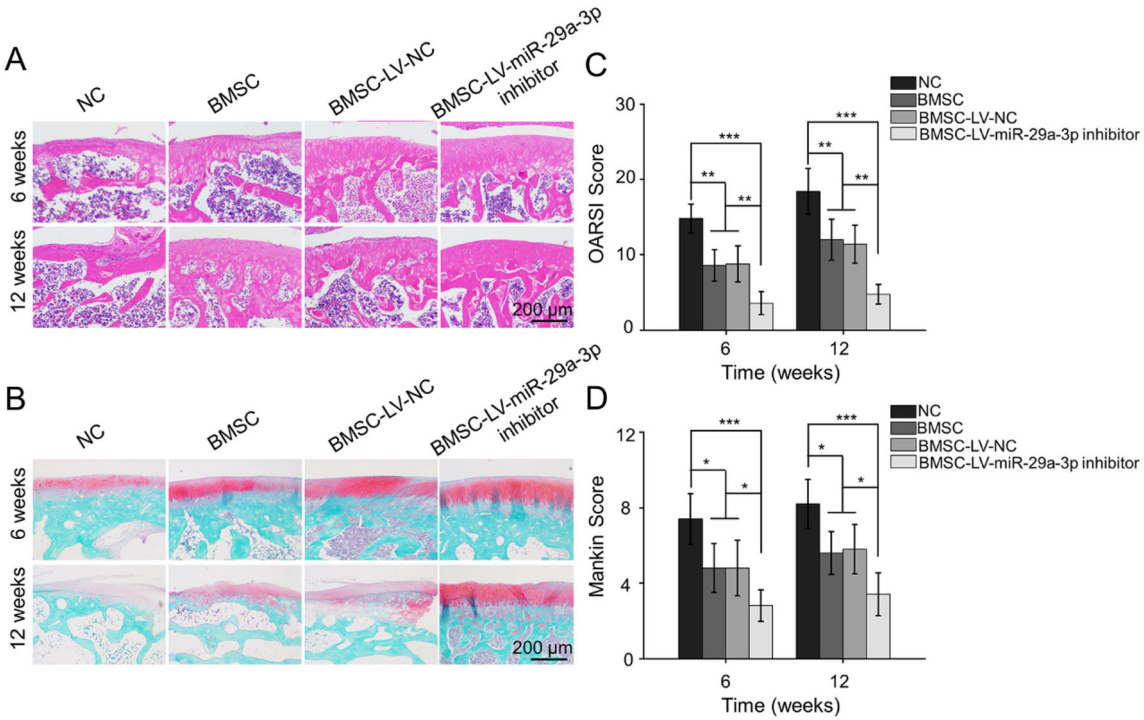

Fig. 4 **A**, **B** H&E and safranin O staining at 6 and 12 weeks after the intra-articular injection. **C**, **D** OARSI scores and Markin scores were statistically analyzed in each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n = 6

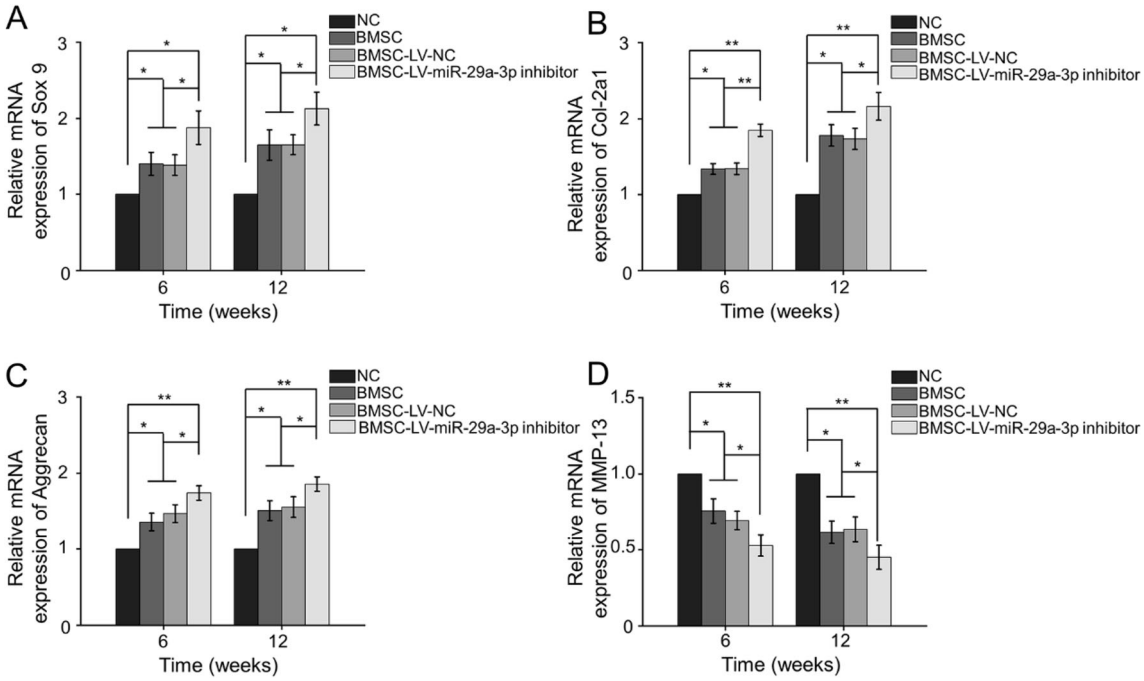

Fig. 5 RT-qPCR analysis of cartilage-related genes of **A** *Sox 9*, **B** *Col-2a1*, **C** *aggrecan*, and **D** *MMP-13* in OA rat model after intra-articular injection. *$p < 0.05$, **$p < 0.01$, n = 6

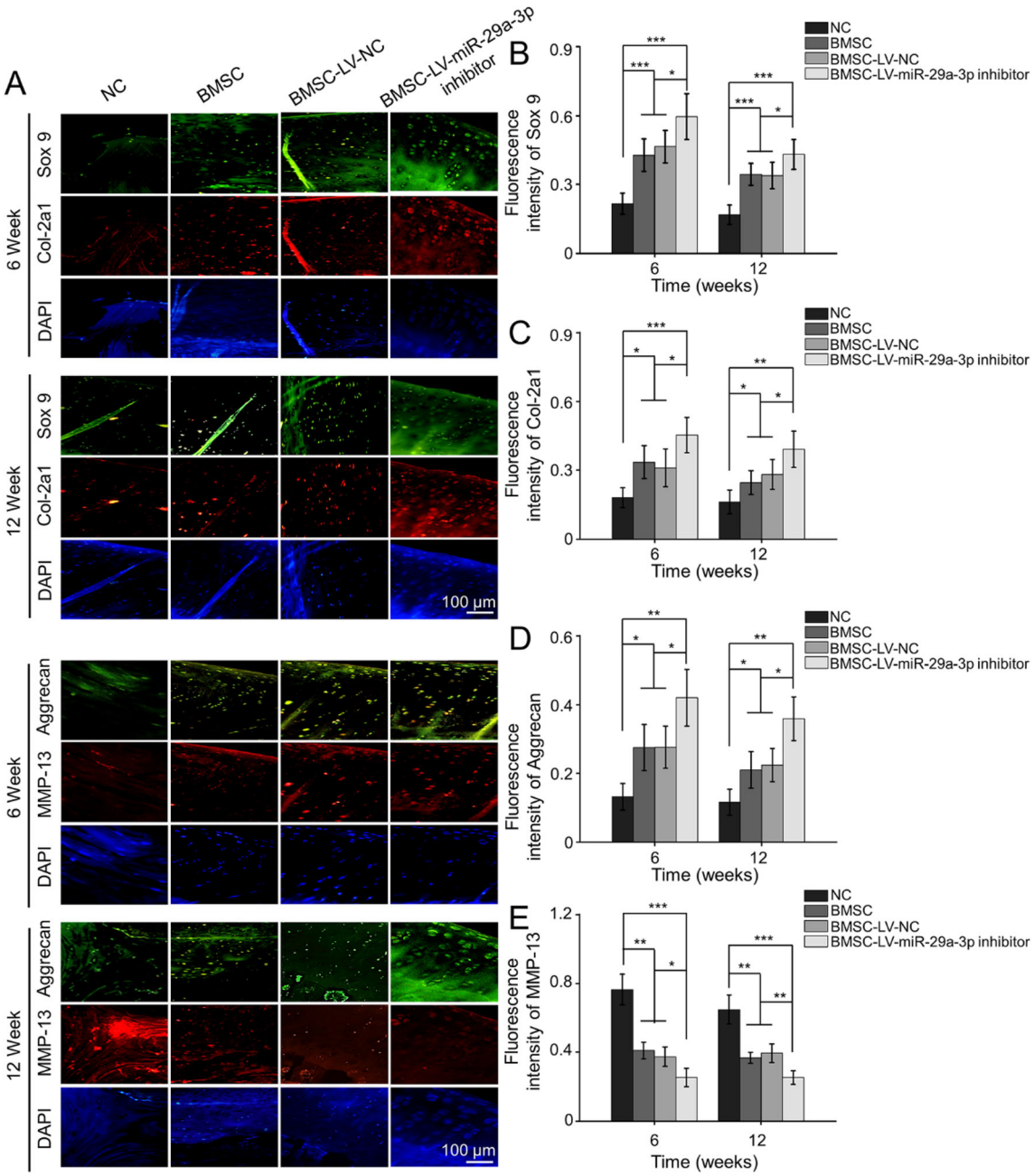

Fig. 6 **A** Representative immunofluorescence images of *Sox 9, Col-2a1, aggrecan, and MMP-13* in cartilage at 6 weeks and 12 weeks post-injection. **B–E** Quantitative statistics of *Sox 9, Col-2a1, aggrecan, and MMP-13* expression. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, n = 6

aggrecan, and *MMP-13* in BMSCs induced by IGF-1. In addition, the efficiency of IGF-1 inducing BMSCs to differentiate into chondrocytes was lower than TGF-β [26]. A new approach targeting IGF-1 to improve its efficacy to induce BMSCs differentiating into chondrocytes may implicate crucial potential.

Therefore, in this study, we used microRNA transfection method to enhance the expression of endogenous IGF-1 and induce chondrogenic differentiation of BMSCs. The results showed that the endogenous overexpression of IGF-1 in BMSCs can significantly induce chondrogenic differentiation, which provides a new strategy for the stem cell therapy of OA.

3.4 BMSCs with miR-29a-3p downregulation prevent cartilage degeneration in OA

The degeneration and destruction of articular cartilage are associated with primary clinical symptoms in the OA joint. In this study, we investigated the potential of miR-29a-3p

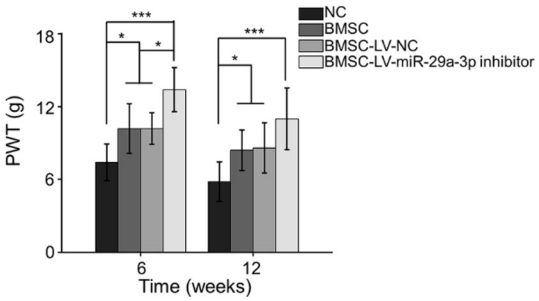

Fig. 7 Paw withdrawal threshold (PWT) was measured to test mechanical allodynia $*p < 0.05$, $***p < 0.001$, n = 6

inhibitor-transfected BMSCs for cartilage regeneration in OA model, which was established by DMM surgery on the knee joints of SD rats.

After 6 and 12 weeks of intra-articular injection, articular cartilages were collected, H&E and safranin O staining were conducted to evaluate the destruction of knee cartilage and scored according to the OARSI grading system and Mankin scoring system by two observers blinded to group information (Fig. 4). As shown in Fig. 4A, the H&E staining indicated that the articular surface in the NC group was not smooth, cartilages missed and defects formed, subchondral bone was exposed, and osteophytes were formed, and a large number of cells on the cartilage surface were necrotic. Observing the cartilage in the BMSC group and BMSC-LV-NC group, it maintained relatively good histological morphology at the 6th week. But at the 12th week, the cartilage layer became thinner and unsmooth, the surface cells on the cartilage had disappeared, and the deep cells were vacuolated. Moreover, the arrangement of chondrocytes in the cartilage layer became loose and irregular, and suspicious fibrous components could be seen on the surface of some cartilage. However, in the BMSC-LV-miR-29a-3p inhibitor group, the articular cartilage was generally intact, with cells being regularly arranged until week 12. Subsequently, safranin O-fast green staining was carried out to observe the cartilage matrix. The results indicated that in the NC group, the cartilage was damaged, and glycoproteins were released in the cartilage, resulting in an uneven distribution of the matrix components and a slight or no coloration of Safranin O. However, in the BMSC-LV-miR-29a-3p inhibitor group, the cartilage had a better morphology and matrix distribution (Fig. 4B). The OARSI cartilage histopathology assessment system and the Mankin scoring system were used to evaluate degenerative status (Fig. 4C, D). The scores in the BMSC-LV-miR-29a-3p inhibitor group were significantly lower than NC group ($p < 0.001$), and BMSC group and BMSC-LV-NC group ($p < 0.05$).

In order to further study the expression of cartilage-related marks in the cartilage, we performed RT-qPCR and immunofluorescence staining to analyze the expression levels of *Sox 9, Col-2a1, aggrecan, and MMP-13* at 6 weeks and 12 weeks post-injection. The transcriptional expression levels of *Sox 9*, *Col-2a1*, *aggrecan* in BMSC-LV-miR-29a-3p inhibitor group were significantly up-regulated compared with NC group, BMSC group, and BMSC-LV-NC group (Fig. 5A–C). In addition, the expression levels of chondrogenic related genes in the BMSC group and BMSC-LV-NC group were also higher than that in the NC group.

Proinflammatory cytokines and MMP show a critical role in the pathogenesis of OA [27]. MMP-13 plays a key role in cartilage remodeling and degradation due to its specificity in cutting Col-2. Previous studies have confirmed that the inflammatory response and MMP-13 activity aggravate OA severity through induction of cartilage degradation [28, 29]. Therefore, the level of MMP was also detected in this study. As displayed in the Fig. 5D, the relative level of *MMP-13* in BMSC-LV-miR-29a-3p inhibitor group was significantly lower than that of NC group ($p < 0.01$), and BMSC group and BMSC-LV-NC group ($p < 0.05$). Subsequently, we performed immunofluorescence staining to detect the expression of *Sox 9, Col-2a1, aggrecan, and MMP-13* in cartilage, and conducted quantitative analysis (Fig. 6). In the BMSC-LV-miR-29a-3p inhibitor group, the fluorescence intensity of *Sox 9, Col-2a1, and aggrecan* were enhanced, and the content of MMP-13 were reduced compared with that in NC group, BMSC group and BMSC-LV-NC group respectively. Although the fluorescence intensity of cartilage related markers in BMSC group and BMSC-LV-NC group was not as good as that in the BMSC-LV-miR-29a-3p inhibitor group, it was also significantly improved compared with that in NC group. The above results revealed miR-29a-3p inhibition could induce BMSCs differentiating into chondrocytes and they filled in the cartilage defects to show the regenerative effect, thus inhibiting cartilage degeneration and reduce OA progression *in vivo*. However, study also has indicated that MSCs has paracrine effect. The growth factors, cytokines, and bioactive substances contained in the microcapsules released from the implanted MSCs can promote the proliferation of chondrocytes and collagen synthesis, which can relieve pain and improve the function of OA patients [30]. At present, the molecular mechanism of BMSCs in cartilage formation is still unclear. In order to avoid fibrocartilage, hypertrophic cartilage and cartilage ossification, it is still necessary to further study the basic research of cartilage-oriented differentiation and expression of MSCs. Herein, the action mechanism of miR-29a-3p inhibitor-transfected BMSCs to reduce OA progression *in vivo* could be a synergy of chondrogenic differentiation of BMSCs induced by IFG-1 overexpression and paracrine

effect of BMSCs to promote the chondrocytes proliferation and collagen synthesis.

3.5 Injection of BMSCs with miR-29a-3p downregulation relieves pain caused by OA

As a kind of degenerative disease, the pain and dysfunction caused by OA greatly perplex the patients. Peripheral pain mechanisms include the direct activation of nociceptors, as well as sensitization of nociceptors by the inflammation in articular cavity [31, 32]. Besides, local immune cells secrete inflammatory cytokines and additional molecular mediators that function on the peripheral nerve terminals of nociceptor neurons [33]. In response to the inflammatory mediators, intracellular signaling pathways lead to a phosphorylation cascade, which reduces the threshold for nociceptor neurons to generate action potentials, eventually resulting in heightened pain sensitivity [34].

As displayed in Fig. 7, the PWT values of NC group, BMSC group, BMSC-LV-NC group, and BMSC-LV-miR-29a-3p inhibitor group were 7.4 ± 1.5, 10.2 ± 2.1, 10.2 ± 1.3, and 13.4 ± 1.8 at 6 weeks, and 5.8 ± 1.6, 8.4 ± 1.7, 8.6 ± 2.1, and 11.0 ± 2.6, at 12 weeks after intra-articular injection, respectively. These results indicated that BMSCs could relieve pain caused by OA, especially with the assistance of miR-29a-3p inhibitor.

4 Conclusion

In conclusion, miR-29a-3p inhibitor transfection induces BMSCs secretion of IGF-1 and stem cell chondrogenic differentiation. For intra-articular injection of BMSCs with miR-29a-3p knockdown, this strategy significantly prevents articular cartilage degeneration and alleviates pain in OA. This suggests miR-29a-3p inhibited BMSCs is a promising strategy to restore cartilage repairing and maintain pain management in the future.

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Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical Statement All animal procedures were performed in accordance with the guidelines for Care and Use of Laboratory Animal Experience and approved by the Animal Care and Use Ethics Committee of Nanjing University of Chinese Medicine (no. ACU191101).

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