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Original Article

miR-205-5p in exosomes divided from chondrogenic mesenchymal stem cells alleviated rheumatoid arthritis via regulating MDM2 in fibroblast-like synoviocytes

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Abstract

Objective: To explore the role and mechanism of chondrogenic bone marrow mesenchymal stem cells (BMSCs)-derived exosomes on Rheumatoid arthritis (RA). **Methods**: The chondrogenesis of BMSCs was induced by chondrogenic medium. Exosomes from BMSCs and chondrogenic BMSCs were isolated and characterized by transmission electron microscope (TEM), laser particle size analyzer and western blot. ELISA was used to analyze the expression levels of pro-inflammatory cytokines and matrix metalloproteinases (MMPs). Western bolt was performed to assess MAPK and NF-κB pathways expression. The inflammation score and the pathological damage of RA mice were evaluated. Luciferase reporter assay and RIP were carried out to examine the relationship between microRNA-205-5p (miR-205-5p) and mouse double minute 2 (MDM2). **Results**: Chondrogenic BMSCs-derived exosomes suppressed pro-inflammatory cytokines, MMPs and MAPK and NF-κB pathways in RA-FLSs. miR-205-5p had a high expression in chondrogenic BMSCs-derived exosomes. Functionally, exosomal miR-205-5p also played the anti-inflammation effects. Besides, MDM2 was a direct target of miR-205-5p. Additionally, chondrogenic BMSCs-secreted exosomal miR-205-5p suppressed the inflammation score, joint destruction, and inflammatory response in collagen-induced arthritis (CIA) mice through MDM2. **Conclusion**: Chondrogenic BMSCs-derived exosomal miR-205-5p suppressed inflammatory response, MAPK and NF-κB pathways through MDM2 in RA, indicating exosomal miR-205-5p might be a potential target for RA treatment.

Keywords: BMSCs, Exosomes, MDM2, miR-205-5p, Rheumatoid Arthritis

Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory immune-mediated disease, which is characterized by synovial tissue inflammation and joint destruction[1,2]. RA has an incidence of 0.5 % to 1 %, with an apparent difference by geographical regions[3]. It was generally considered that the risk of RA mainly depended on genetic factors with heritability is currently estimated as 40-65%[4]. The etiology and specific mechanism of RA are still unclear. Studies have shown that the pathogenesis of RA involves many different pathways. The typical pathological characteristic of RA is the obvious thickening of synovial membrane caused by the increased number of fibroblast-like synoviocytes (FLSs)[5]. The proliferation of FLSs induces the secretory

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accumulation of proinflammatory cytokines that expedite the degradation of the extracellular matrix and cartilage, and continue to enhance FLSs proliferation[6,7]. As the major theme, inflammatory cytokines, such as interleukins (ILs), tumor necrosis factor-α (TNF-α), and their receptor play an irreplaceable function in RA pathogenesis[8,9].

Mesenchymal stem cells (MSCs) have the ability of self-renewal and multidirectional differentiation, which are characterized by low immunogenicity[10]. The therapeutic efficacy of MSCs was based on the ability of MSCs to migrate to the damaged tissues and differentiate into precursor cells with therapeutic potential, thereby delivering therapeutic effects[11]. MSCs exhibit diverse function in immune regulation. As a potential treatment method of RA, MSCs have been reported to provide clinical benefits for patients with active RA[12,13]. Though a lot of researches focused on crosstalk between MSCs and different cell types, it was unclear now that how MSCs act a role in RA process[14]. A deep understanding of the regulation mechanism of MSCs still has urgent requirements.

Exosomes, as a kind of bioactive substance of MSCs paracrine, have become a research hotspot in recent years. Exosomes are vesicles that can be secreted from any type of cell. The contents of exosomes, including cytokines, lipids, mRNAs and miRNAs, play important roles in intracellular signaling and intercellular communication[15]. Exosomes derived from MSCs have been widely applied in the treatment of several diseases, such as acute kidney injury[16,17], Alzheimer's disease[18], heart failure[19], liver fibrosis and pulmonary hypertension[20,21], instead of using MSCs directly. Bone marrow-derived MSCs (BMSCs) derived exosomal miRNAs affect RA development by targeting specific proteins to regulate multiple signaling pathways. Therefore, MSCs derived exosomal miRNAs are potential biomarkers for novel cell-free therapies for RA. The aim of this study was to explore the role and mechanism of chondrogenic BMSCs-derived exosomes on RA.

Materials and Methods

Cell culture

BMSCs derived from C57BL/6 mice were purchased from the American Type Culture Collection and cultured in α-modified Eagle medium (Gibco, NY, USA) supplemented with 15% fetal bovine serum (FBS, Gibco, NY, USA) with 5% CO_2 at 37°C.

Isolation of FLSs

The mouse FLSs were isolated according to the reported method[22]. In brief, the menisci of the joints with adjacent synovial membrane were separated and minced into small sections (1-3 mm). The small sections were plated in cell culture dishes (10 cm) in DMEM (Gibco, NY, USA) complemented with 15% FBS (Gibco, NY, USA), 100 U·mL^{-1} penicillin (Sigma-Aldrich, St. Louis, MO), 100 μg·mL^{-1} streptomycin (Sigma-Aldrich, St. Louis, MO) and 2.5 mmol·L^{-1} L-glutamine (Sigma-Aldrich, St. Louis, MO) for 14 days. Then FLSs were collected, passage culture for 3–5 times, and used for the following experiments.

Chondrogenesis in vitro

BMSCs at passages 3-6 were dissociated for single-cell suspension stating. 10 μL droplets per well, BMSCs at a density of 1×10^7 cells.mL^{-1}, were seeded and allowed to form cell aggregates. Following the incubation for 24 h, BMSCs were cultured in a chondrogenic medium consisted of high glucose DMEM (Gibco, NY, USA), 1×GlutaMax (Thermo Scientific, MA, USA), 100 nM dexamethasone (Sigma-Aldrich, St. Louis, MO), 200 μM ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, MO), 100 μM sodium pyruvate (Thermo Scientific, MA, USA), 40 μg/mL L-proline (Sigma-Aldrich, St. Louis, MO), 1% ITS-X (Thermo Scientific, MA, USA) and 100 U/mL PenStrep (Thermo Scientific, MA, USA) and 10 ng/mL TGF-β1 Abcam, Cambridge, UK) with 5% CO_2 at 37°C for another 24 h[23]. Differentiation was performed for 14 days.

Isolation and identification of exosomes

BMSCs-exosomes were prepared from the culture medium as previously described[24]. Briefly, the culture medium was gathered to centrifugation at 2000 g for 30 minutes. Total Exosome Isolation (from cell culture medium) (4478359, Thermo Scientific, MA, USA) was added (1:0.5, v/v) into the media. The mixture was performed homogenous solution, and then incubated at 4°C. After incubation, centrifuge at 10000 g for 70 min at 4°C and discard the supernatant. Resuspend the pellet by PBS (about 1% volume of origin media) and prepare for identification. The two important indicators for exosomes, such as the concentration and size distribution, were measured using MasterSizer 2000 system (Malvern Panalytical, Malvern, UK). Exosome morphologies were observed under JEM-2100 TEM (JEOL, Japan). The exosome-associated markers CD9 (ab92726, Abcam, Cambridge, UK) and CD63 (sc-5275, Santa Cruz Biotechnology Inc., CA, USA) were detected by Western blot analysis.

Isolation of RNA

Total RNA was isolated from cells using a total RNA extraction kit (K156002, Invitrogen, CA, USA) according to the manufacturer's guidelines. Total RNA was isolated from BMSCs-exosomes and chondrogenic BMSCs-exosomes using a total exosome RNA isolation kit (4478545, Invitrogen, CA, USA) according to the manufacturer's guidelines. The RNA concentrations were detected by NanoDrop (Thermo Scientific, MA, USA). About 500 ng RNA was reverse transcribed into cDNA with PrimeScript RT Enzyme Mix (DRR037A, TaKaRaBio, Beijing, China) according to the manufacturer's guidelines.

Table 1. Primer sequences used in RT-qPCR.

Target gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
miR-126-3p	UCGUACCGUGAGUAAUAAUGCG	CAUUAUUACUCACGGUACGAUU
miR-205-5p	CGTCCTTCATTCCACCGG	AGTGCAGGGTCCGAGGTATT
miR-29b-3p	TGCGGTAGCACCATTTGAAAT	CCAGTGCAGGGTCCGAGGT
U6	CCGTATGACCTCCTTCCACAGA	TCTGTCCACCTCTGAAACCAGG
MDM2	TTAGAGCACCCTGTCACCACG	GTTACCATCATAAGCCTACAGACCYAC
GAPDH	ATTCAACGGCACAGTCAA	CCTGGAAGATGGTGATGG

RT-qPCR

The primer sequences used in RT-qPCR were shown in Table 1 (Genscript, Nanjing, China). RT-qPCR was carried out using the SYBR® Premix Ex TaqTM kit (Bio-Rad, CA, USA) on an ABI 7000 Thermocycler (Applied Biosystems, MA, USA). GAPDH was used as the internal control for mRNA expression. The $2^{-\Delta\Delta CT}$ method was used to analyze the results.

ELISA

The method used for measuring the cytokine and matrix metalloproteinase has been previously published[25]. The levels of IL-1β, IL-6, tumor necrosis factor α (TNF-α) and MMP-1 and MMP-13 in serum or cell lysates were measured using specific Quantikine ELISA Kits (R&D System, Chaska, MN, USA).

Western blot

Proteins from the cells and tissues were lysed by ice-cold radioimmunoprecipitation assay (RIPA) buffer and determined by a BCA protein assay kit (23229, Thermo Scientific, MA, USA). A quantity of 20–30 μg of lysates was subjected to a 10% SDS-PAGE and transferred to a PVDF membrane. Blots were probed with the primary antibody (Abcam) for the indicated proteins, such as Collagen II, Aggrecan, Sox9, Smad1/4, p-Smad1/4, p38, p-p38, ERK, p-ERK, JNK, p-JNK, NF-κB-p65, p-IκBα, and GAPDH overnight at 4°C. After washing, the bands were incubated with the appropriate HRP-conjugated secondary antibody (ab6721 or ab6728, Abcam, Cambridge, UK), which were subsequently detected with an enhanced chemiluminescence kit (P1052, Applygen Technologies, Beijing, China).

Cell transfection

MiR-205-5p mimic and its scrambled control (miR-NC mimic), miR-205-5p inhibitor and inhibitor control (miR-NC inhibitor) and were purchased from GenePharma Co. Ltd. (Shanghai, China). The sequences of the oligonucleotides used were shown in Table 2. FLSs (4×10^5 cells per well) were seeded into 6-well plates and cultured as described above. Cells were transfected by Lipofectamine™ 2000 CD Transfection Reagent (12566014, Thermo Scientific, MA, USA) according to the instructions. The final concentrations of miRNAs were 50 nM, and were transfected into cells using 0.25 % lipofectamine 2000 for 48 h at 37°C.

Table 2. Oligonucleotides used in transfection.

Oligonucleotides	Sequence (5'-3')
miR-205-5p mimic	5'-UCCUUCAUUCCACCGGAGUCUG-3'
miR-NC mimic	5'-UCGCUUGGUGCAGGUCGGGAA-3'
miR-205-5p inhibitor	5'-CAGACUCCGGUGGAAUGAAGGA-3'
miR-NC inhibitor	5'-CAGUACUUUUGUGUAGUACAA-3'

3'UTR luciferase reporter assay

FLSs (4×10^5 cells per well) were seeded into 6-well plates and cultured as described above. The bioinformatics analysis softwares, such as miRwalk, targetscan, miRDB, miRTarbase were used to predict the genes (such as MDM2) targeting with miR-205-5p. The putative binding sites of miR-205-5p on the MDM2 3'-UTR were predicted using the TargetScan 7.0 (http://www.targetscan.org/vert_70/) online tool. The MDM2 3'-UTR sequences were introduced into the luciferase reporter vector (pGL3-Basic) to construct wild-type (WT) luciferase reporter plasmids (MDM2 WT), and were mutated to construct mutant (MUT) luciferase reporter plasmids (MDM2 MUT). FLSs were co-transfected with 0.5 μg/μl luciferase reporter plasmids, 50 nM miR-489 mimics or inhibitors, and their negative control using Lipofectamine™ 2000 CD Transfection Reagent (12566014, Thermo Scientific, MA, USA). After 48 h, cells were collected and measured using a luciferase reporter assay system (Promega, WI, USA). The dual-luciferase activity of the target gene was normalized to Renilla luciferase activity. The reported data represent the average of three independent transfection experiments performed in triplicate.

RIP assay

Protein A/G Magnetic Beads (Thermo Scientific, Waltham, MA, USA) were used to conduct RIP assay. We transfected

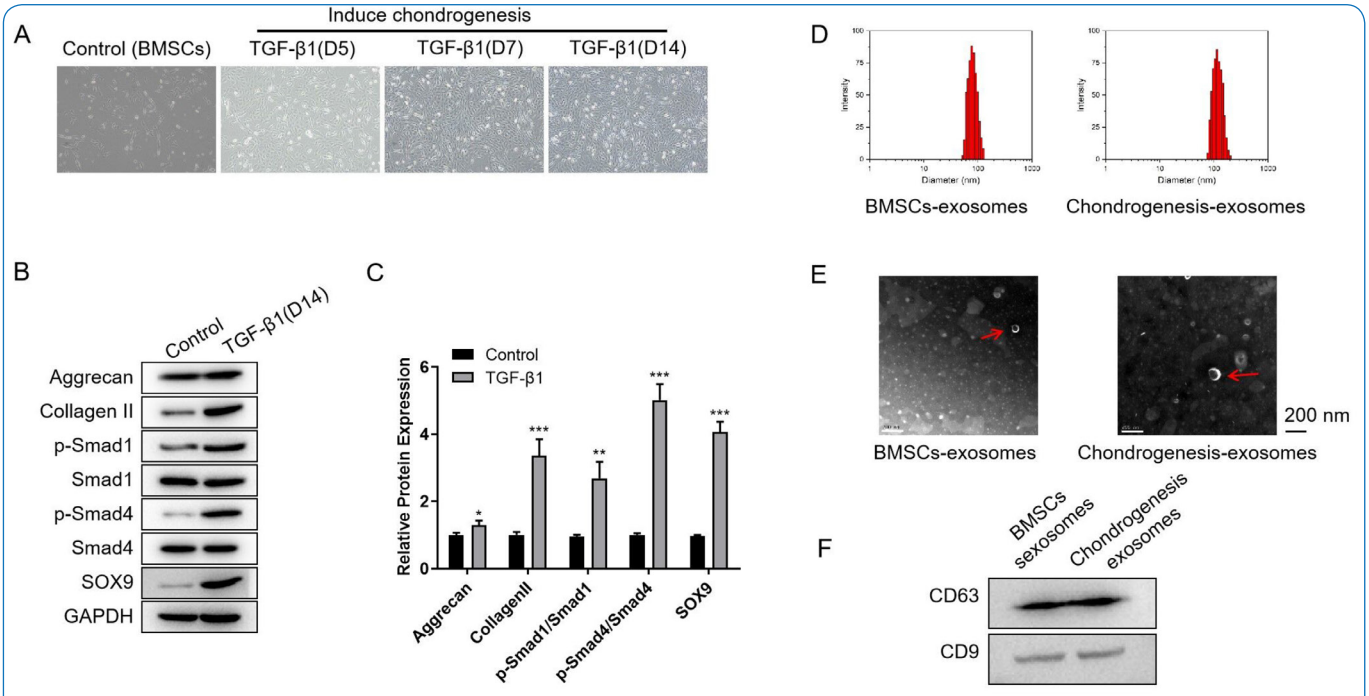

Figure 1. Cultivation and characterization of exosomes divided from chondrogenic BMSCs. (A) The morphological images of BMSCs and BMSCs induced with the TGF-β1 at several points (5, 7 and 14 days) were observed under microscope. (B) The protein expressions of collage II, Smad1, p-Smad1, Smad4, p-Smad4, SOX9, and Aggrecan were assessed with western blot assay. (C) Statistical analysis results of western blot. (D) The diameters for BMSCs secreted exosomes (BMSCs-exosomes) and chondrogenic BMSCs secreted exosomes (Chondrogenesis-exosomes) were tracked by Nanosight tracking analysis. (E) The shape and structure of exosomes were observed by transmission electron microscopy. Exosomes were indicated by a red arrow. Scale bar: 200 nm. (F) The protein expressions (CD63 and CD9) in the exosomes were detected by western blot. The values shown are the means ± SEM from three independent experiments. **p<0.01 and ***p<0.001, were indicated as the difference compared with the control groups.

miR-205-5p mimic and inhibitor into FLSs. Then, cells were lysed in RIPA and incubated with magnetic beads, Ago2 antibodies or IgG at 4°C overnight. Subsequently, RNA was extracted and then analyzed by RT-qPCR.

Collagen-induced arthritis (CIA) mouse model

8-week-old C57BL/6 mice (male, 20 g ± 2 g) were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). The experimental protocol was in accordance with the US National Institutes of Health's Guidelines of Laboratory Animal Use. All animal experiments were approved by the Animal Ethics Committee of The Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine.

C57BL/6 male mice (n=24) were randomly divided into four groups: Normal group (n=6), CIA group (n=6), CIA+exo-miR-NC group (n=6) and CIA+exo-miR-205-5p group (n=6). CIA mouse model was generated by type II collagen induction according to the reported method[26]. Briefly, 2 mg/ml of bovine type II collagen (Chondrex, WA, USA) was emulsified with complete Freund's adjuvant (CFA, Chondrex, WA, USA) containing heat-killed Mycobacterium tuberculosis H37Ra (1:1 v/v). Each mouse was given a 100 μl of type II collagen emulsion by intradermal injection into the tails for 21 days. Mice in normal group were only injected with an equal volume of PBS. Exosomes were separated from chondrogenic BMSCs, and transfected with miR-NC mimic or miR-205-5p mimic. Then, mice were intradermally injected 200 μl exosomes carrying miR-NC or miR-205-5p into the tails two times per week for 14 days.

Hematoxylin & eosin (HE) analysis

The whole knee joints of mice were collected, fixed in 10% neutral formalin for 4 days, and decalcified with EDTA (0.2 M, pH=7.4) for 4 weeks. The knees were sectioned in frozen to 5 μm sections, and stained with hematoxylin to show the nucleus and eosin to observe the symptoms of the joints for the mice in all groups, according to the reference[27].

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 software. Data were demonstrated as means ± standard deviation (SD). Two-tailed Student's t-test and one-way ANOVA

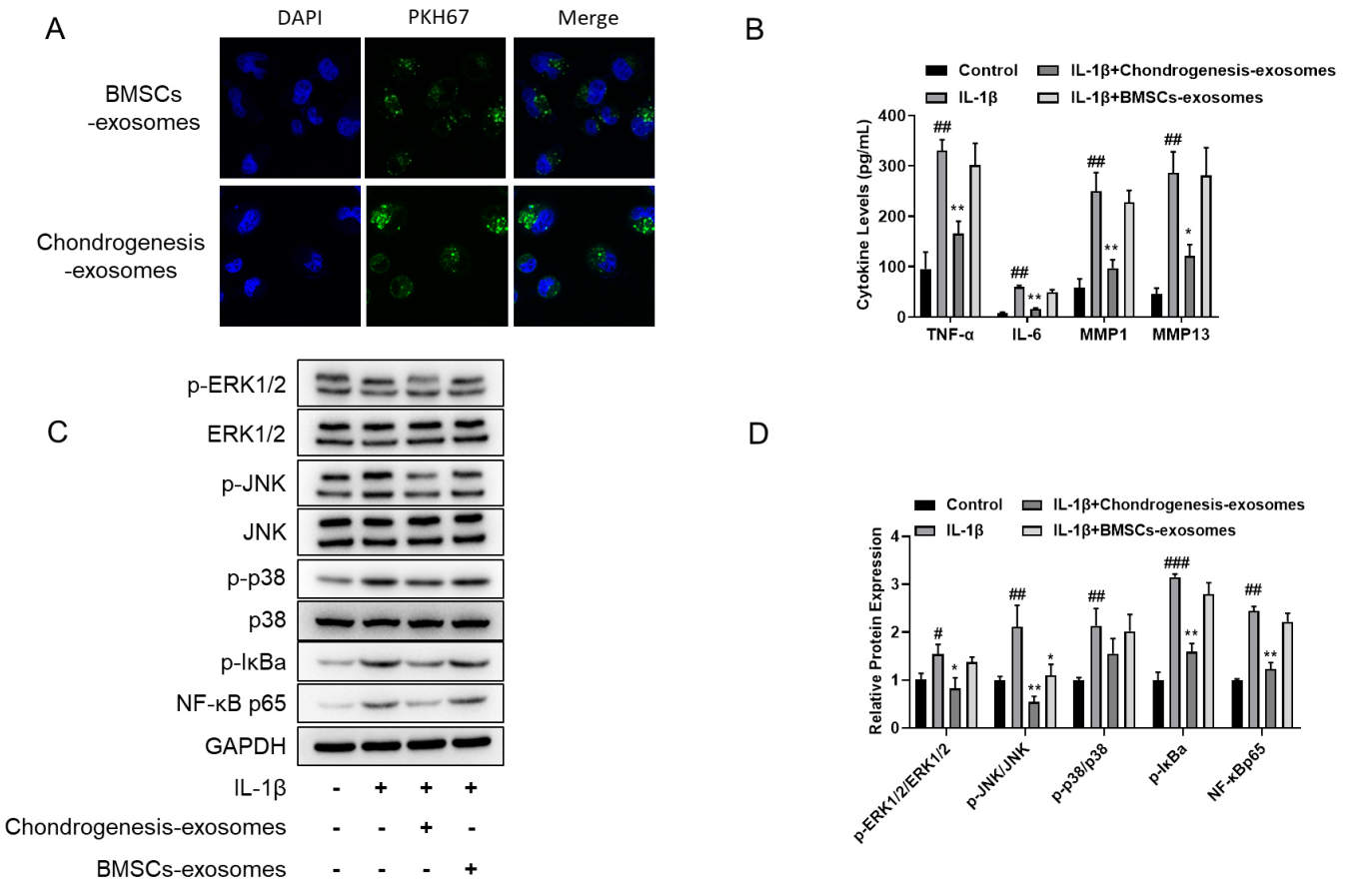

Figure 2. Chondrogenic BMSCs secreted exosomes inhibited inflammation in IL-1β-treated RA-FLSs. RA-FLSs were divided into Control, IL-1β, IL-1β+Chondrogenesis-exosomes, IL-1β+BMSCs-exosomes groups. (A) Representative fluorescence images of PKH67-labeled exosomes were englobed by FLSs. (B) The expression of pro-inflammatory cytokines and MMPs in RA-FLSs were evaluated by ELISA. (C-D) The related protein expressions were assessed by western blot. Chondrogenic BMSCs exosomes suppressed the activation of MAPK and NF-κB signal pathway in IL-1β treated FLSs. Data are revealed as the mean ± SD obtained from three independent experiments. $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ were indicated as the difference compared with control group;*p<0.05 and **p< 0.01 were indicated as the difference compared with IL-1β group.

with Turkey's test were performed to compare the difference among two or multiple groups, respectively. Data with *p* values smaller than 0.05 was considered significant difference.

Results

Cultivation and characterization of exosomes divided from chondrogenic BMSCs

As shown in Figure 1A, undifferentiated BMSCs grew polygonal adherent as control. After TGF-β1 addition, the morphology of BMSCs was significantly changed, and the size and shape of BMSCs stage gradually showed a uniform pebble shape in the chondrogenesis (Figure 1A). Western blot assay showed that the protein levels of chondrogenic specific genes including Aggrecan, Collagen II, SOX9, phosphorylation of Samd1 and Samd4 were significantly higher in TGF-β1 group compared with control group (Figure 1B and 1C), suggesting that the chondrogenic status of BMSCs.

Next, exosomes were isolated from the medium of undifferentiated BMSCs (BMSCs-exosomes) and chondrogenic BMSCs (Chondrogenesis-exosomes). Laser particle size analyzer and TEM showed that the particles were in size around 100 nm, and were the cup/round-shaped morphology, regardless of chondrogenic differentiation or not (Figure 1D and 1E). Western blot assay showed that isolated exosomes were highly expressed exosomal markers (CD63 and CD9) (Figure 1F). These results suggested that the size and morphology were similar between exosomes divided from chondrogenic BMSCs and undifferentiated BMSCs.

Exosomes inhibited inflammation in IL-1β-treated RA-FLSs

To determine the role of exosomes in RA, FLSs were treated by IL-1β (10 ng/mL) to construct the RA cell model.

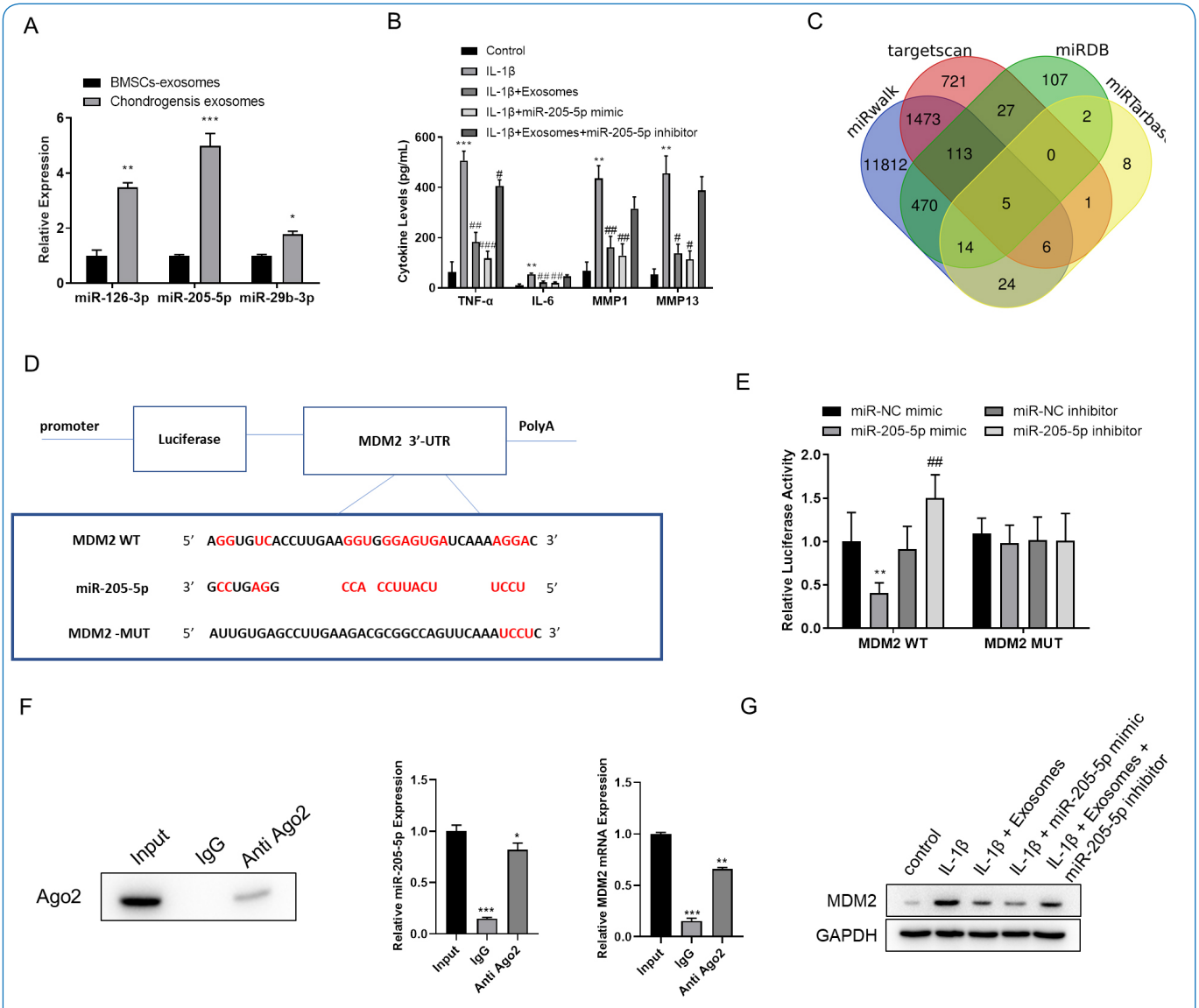

Figure 3. Anti-inflammation effects depend on miR-205-5p in exosomes binding to the 3'UTR of MDM2. (A) The relative miRNA expressions, such as miR-205-5p, miR-126-3p and miR-29b-3p, were detected by qRT-PCR. (B) Cytokine levels were tested in FLSs by ELISA. (C) The target genes regulated by miRNA-205-5p were predicted by bioinformatics databases. (D) TargetScan prediction tool showed the potential binding sites for miR-205-5p and 3′-UTR of MDM2. (E) Dual-luciferase reporter gene assay confirmed the binding sites between miR-205-5p and MDM2. (F) RIP assay was executed in FLSs after transfection. And the miR-205-5p and MDM2 expressions were assessed by qRT-PCR. (G) The protein levels of MDM2 were detected in FLSs. The values are presented as the means ± SD of three individual experiments. In Figure 3A, *p<0.05, **p<0.01 and ***p<0.001 were indicated as the difference compared with BMSCs-exosomes group. In Figure 3B, *p<0.05 and **p<0.01 were indicated as the difference compared with control group; #p<0.05, ##p<0.01 and ###p<0.001 were indicated as the difference compared with IL-1β group. In Figure 3E, **p<0.01 and ##p<0.01 were indicated as the difference compared with NC mimic group and NC inhibitor group, respectively.

PKH67 labeled BMSCs-derived exosomes and chondrogenic BMSCs-derived exosomes were co-cultured with FLSs. Fluorescence microscopy showed that both BMSCs-derived exosomes and chondrogenic BMSCs-derived exosomes were absorbed by FLSs (Figure 2A). These results indicated exosomes could play important roles on FLSs. ELISA showed that the expression levels of pro-inflammatory cytokines, including TNF-α, IL-6 and MMPs including MMP1, MMP13 in FLSs were significantly upregulated by stimulation of IL-1β, which were suppressed remarkably if treated with exosomes secreted from chondrogenic BMSCs, but not BMSCs-exosomes (Figure 2B). To investigate the signaling pathways involved in inflammation, the activation status of both MAPK and NF-κB were evaluated. The western blot results

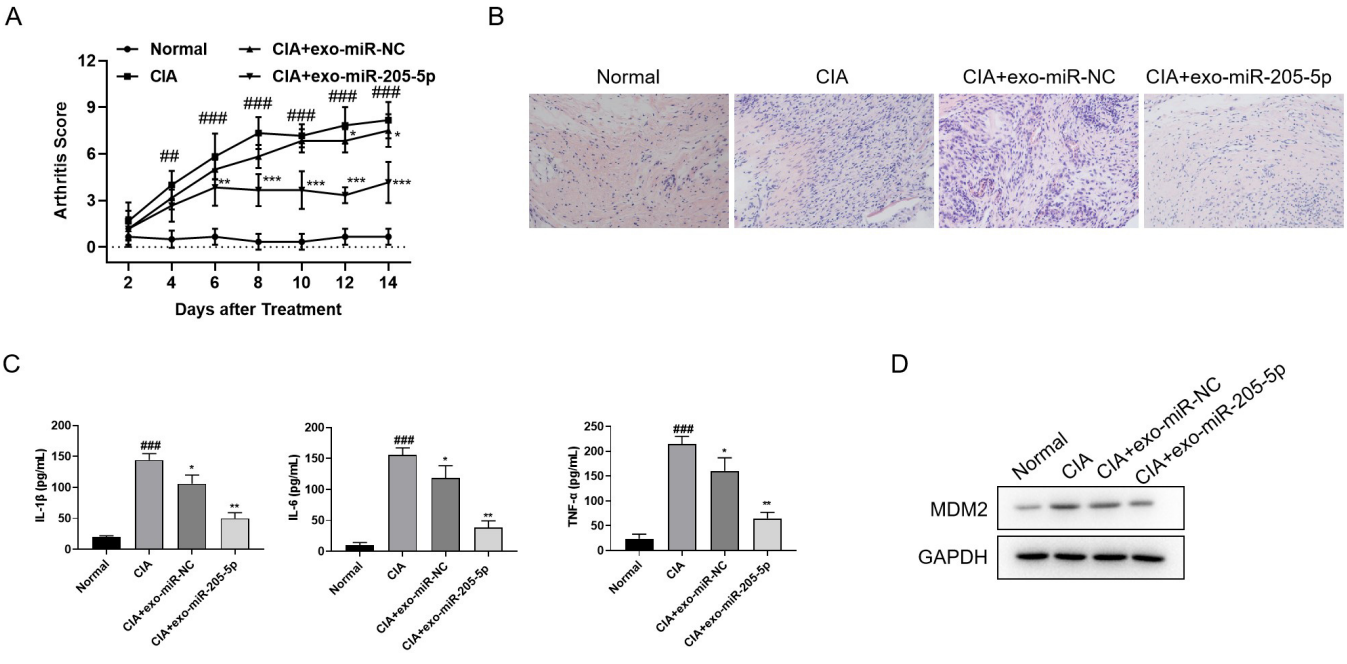

Figure 4. Exo-miR-205-5p suppressed inflammatory response in CIA mice. (A) The inflammation scores for mice in Normal, CIA, CIA+Exo-miR-NC and CIA+Exo-miR-205-5p group. (B) H&E staining results showed for the pathological damage of the joint for mice. (C) The levels of IL-1β, IL-6 and TNF-α in the serum of all group were detected by ELISA. (D) The protein expression of MDM2 was measured in CIA mice. All the experiments were independently performed in triplicates. ###p<0.001 was indicated as the difference compared with the normal groups; *p<0.05, **p<0.01 was indicated as the difference compared with CIA group.

showed that chondrogenic BMSCs exosomes inhibited the phosphorylation of ERK1/2, JNK and p38 in RA-FLSs, and the activation of NF-κB p65 was similar to that of MAPKs (Figure 2C and 2D). Taken together, these data demonstrated that exosomes in chondrogenic BMSCs could inhibit inflammation through both MAPK and NF-κB signaling pathways.

Anti-inflammation effects depend on miR-205-5p from chondrogenic BMSCs exosomes to bind the 3'UTR of MDM2 mRNA

To search whether chondrogenic BMSCs-derived exosomes are also involved in mediating RA progress through exosomal miRNAs. Through a large number of literature surveys, we screened out 3 miRNAs that were abnormally expressed in chondrogenic BMSCs exosomes and related to inflammation (miR-29b-3p, miR-205-5p, miR-126-3p). The results showed that miR-205-5p had a high expression in exosomes from chondrogenic BMSCs (Figure 3A). Therefore, miR-205-5p was chosen for subsequent studies. To investigate whether chondrogenic BMSCs derived exosomes inhibited RA-FLSs inflammation due to miR-205-5p, miR-205-5p mimic/inhibitor and their control were synthesized and transfected into IL-1β-treated RA-FLSs. The results showed that miR-205-5p overexpression had similar anti-inflammatory effects to chondrogenic exosomes, while miR-205-5p inhibitors reversed the effects of chondrogenic BMSCs derived exosomes (Figure 3B). These results indicated that miR-205-5p might act an important function in BMSC therapy. We used bioinformatics analysis software such as miRwalk, targetscan, miRDB, miRTarbase to predict the genes targeting with miR-205-5p. The results were intersected to screen out 5 target genes (MDM2 RUNX2 ZEB2 DDX5 LRRK2) (Figure 3C). MDM2 was used for further study due to MDM2 promoted RA via activating the MAPK and NF-κB[28]. Furthermore, sequence analysis suggested that miR-205-5p may bind to the 3' UTR of MDM2 (Figure 3D). We further conducted luciferase assay to examine the target relationship by transfecting MDM2-3'UTR fragment luciferase (WT) and mutation fragment luciferase (MUT), respectively. The luciferase signals were verified that at present of miR-205-5p mimic, it had a decrease when there was a transfection with WT fragment, while the MUT fragment was not (Figure 3E). We also verified their interaction through the RIP experiment, MDM2 mRNA and miR-92a-3p could be detected by RT-qPCR from the complex with Ago2 protein, which separated by Ago2 antibody and beads, nor IgG (Figure 3F). In addition, miR-205-5p mimic was associated with downregulated expression of MDM2 protein level, which was inversed by the inhibition of miR-205-5p. As evaluated by western blot (Figure 3G), chondrogenic BMSCs exosomes

showed anti-inflammation effects depend on miR-205-5p bind to the 3'UTR of MDM2 mRNA.

Exo-miR-205-5p suppressed inflammatory response in CIA mice through downregulation of MDM2

To explore the therapeutic potential of chondrogenic BMSCs secreted exosomal miR-205-5p, we next examined its capacity to suppress inflammatory in CIA mice. The inflammation scores of CIA were recorded every 2 days. A significant elevation of average inflammation score was observed in CIA mice compared with the normal mice (Figure 4A), which was decreased by chondrogenic BMSCs secreted exosomes. Furthermore, the inhibition of inflammation in CIA+exo-miR-205-5p group was much higher than that in CIA+exo-miR-NC group. Also, the histological analysis was determined by H&E. As shown in Figure 4B, CIA mice showed significant leucocyte infiltration in the joints. However, a significant reduction for inflammation was found in CIA mice with the exo-miR-NC or exo-miR-205-5p treatment. The proinflammatory cytokines levels in serum were assessed by ELISA. The results showed that CIA significantly elevated the levels of IL-1β, IL-6 and TNF-α in serum, while there was a significant reduction in both CIA+exo-miR-NC and CIA+exo-miR-205-5p group (Figure 4C). In particular, when the miR-205-5p was overexpressed in the chondrogenic BMSCs secreted exosomes, the inhibition of the levels of inflammatory factors was more obvious. Western blot results showed that exo-miR-205-5p inhibited in exo-miR-205-5p delayed the inflammatory response in CIA mice through downregulation of MDM2 (Figure 4D).

Discussion

MSCs play a therapeutic role mainly through paracrine signaling pathways, and their exosomes have a variety of biological activities, and are considered to be an ideal source of exosomes[29]. It has been reported that MSCs secreted exosomes play an important role in ameliorating type 2 diabetes, mediating cartilage repair and regenerating bone[30-32]. It has been proved that miRNAs are important components in transferring to recipient cells and mediating a lot of biological processes. It has been reported that the expression of miRNA from exosomes, which were derived by hMSCs with and without chondrogenic induction, has been compared and the results show that there are significant differences between these exosome components, which may affect the therapeutic efficacy of BMSCs[33]. In this study, miR-205-5p was one of these components that have a great change of level in chondrogenic BMSCs, some researchers have revealed that miR-205-5p may be related to proliferation and apoptosis in many diseases, such as cancer, osteoporosis and others[34-36].

NF-κB is a considerable transcription factor, which is related to cell proliferation, differentiation and cytokine expression[37]. Especially, NF-κB acts an essential function in mediating immune and inflammatory response[38]. MAPK is another signaling that has been considered closely related to RA[39]. In the current research, IL-1β induced the inflammatory cytokines expression, such as TNF-α and IL-6 in synovial. Chondrogenic BMSCs exosomes inhibited the NF-κB and MAPK pathways.

MDM2, a nuclear-localized E3 ubiquitin ligase, was first discovered in p53-mediated cell cycle arrest and apoptosis[40]. The regulation function of MDM2 on inflammatory activity and lupus nephritis indicated its relationship with inflammation[41,42]. MDM2 inhibitor, Nutlin-3a, was reported could alleviate RA in CIA mice via MAPK and NF-κB pathways and it consistent with the results of exosomes in our research[43]. Taken results by bioinformatics analysis into consideration, it could assume that MDM2 may target the miR-205-5p in chondrogenic BMSCs exosomes. Subsequent cell transfections and luciferase reporter assay confirmed that miR-205-5p bound to the 3'UTR of MDM2 mRNA, and further RIP assay verified this binding relationship.

CIA model, similar to human RA, was the most commonly used one in various animal models for RA research[44]. It could be clearly observed that red swelling in the paws, inflammatory cell infiltration, and joint deformation in most of CIA mice. In the present study, CIA mice had a significant elevation of average inflammation score. Zhang J. et al. revealed that BMSCs-derived exosomes inhibited the average inflammation score after 16 days treatment[45]. In the present study, chondrogenic BMSCs-derived exosomes suppressed the average inflammation score after 12 days treatment. These results indicated that chondrogenic BMSCs-derived exosomes might have better treatment effect than BMSCs-derived exosomes. Furthermore, chondrogenic BMSCs-derived exosomal miR-205-5p suppressed the average inflammation score after 6 days treatment. These symptoms evidently alleviated in mice with exo-miR-205-5p treatment, combined with the histological analysis and serum analysis, the therapeutic potential of miR-205-5p has been clearly demonstrated. The objective in our work was to analyze the function of chondrogenic BMSCs-derived exosomes and carrying exosomal miR-205-5p (exo-miR-205-5p) in RA, with the ultimate goal to evaluate their therapeutic efficacy. Our work validated the role of exo-miR-205-5p secreted by chondrogenic BMSCs in RA process *in vitro*, and showed that miR-205-5p is an inflammatory inhibitor by targeting MDM2, which is a key regulator of NF-κB and MAPK pathways. Finally, CIA model in mice was established to observe the effect of exo-miR-205-5p *in vivo*. Our study provides evidence of a novel functional association between miR-205-5p and the proto-oncogene MDM2, which may provide insights into the future development of BMSCS-derived exosomes that have a selective advantage over single injection of BMSCs. The present study sheds light on the therapeutic potential of chondrogenic BMSCs-derived exosomes in RA.

Conclusion

Taken together, to the best of our knowledge, the present study was the first to explore the roles of chondrogenic BMSCs-derived exosomes on RA. Our study demonstrated that chondrogenic BMSCs-derived exosomes regulate MAPK and NF-κB pathways to affect the inflammatory expression of RA through miR-205-5p/MDM2 axis, providing new insights for RA treatment. However, how to apply safely chondrogenic BMSCs-derived exosomal miR-205-5p for clinical RA treatment needs further research, especially in animal models before employment in clinic trials. Therefore, further research will be in-depth study with an aim toward eventual translational research.

Authors' contributions

Conceptualization: Wukai Ma; Data curation: Fang Tang; Formal analysis: Lina Xiao; Funding acquisition: Shan Han; Investigation: Xueming Yao; Methodology: Qiongyu Zhang; Project administration: Jiayan Zhou; Resources: Yanjun Wang; Writing - original draft: Wukai Ma; Writing - review & editing: Wukai Ma and Lina Xiao. All authors read and approved the final manuscript.

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