Exosomes from adipose-derived stem cells promote chondrogenesis and suppress inflammation by upregulating miR-145 and miR-221

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Abstract. Osteoarthritis (OA) is one of the most prevalent joint disorders globally. Patients suffering from OA are often obese and adiposity is linked to chronic inflammation. In the present study, the potential of using exosomes isolated from adipose-derived stem cells (ADSCs) as a therapeutic tool for reducing chronic inflammation and promoting chondrogenesis was investigated using patient-derived primary cells. First, it was tested whether patient-derived ADSCs could differentiate into chondrogenic and osteogenic lineages. The ADSCs were then used as a source of exosomes. It was found that exosomes isolated from ADSCs, when co-cultured with activated synovial fibroblasts, downregulated the expression of pro-inflammatory markers interleukin (IL)-6, NF-κB and tumor necrosis factor-α, while they upregulated the expression of the anti-inflammatory cytokine IL-10; without exosomes, the opposite observations were made. In addition, inflammation-inflicted oxidative stress was induced *in vitro* by stimulating chondrocytes with H_2O_2. Treatment with exosomes protected articular chondrocytes from H_2O_2-induced apoptosis. Furthermore, exosome treatment promoted chondrogenesis in periosteal cells and increased chondrogenic markers, including Collagen type II and β-catenin; inhibition of Wnt/β-catenin, using the antagonist ICG-001, prevented exosome-induced chondrogenesis. Periosteal cells treated with exosomes exhibited higher levels of microRNA (miR)-145 and miR-221. The upregulation of miR-145 and miR-221 was associated with the enhanced proliferation of periosteal cells and chondrogenic potential, respectively. The present study provided evidence in support for the use of patient-derived exosomes, produced from ADSCs, for potential chondrogenic regeneration and subsequent amelioration of osteoarthritis.

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Introduction

Osteoarthritis (OA) is the most prevalent joint disorder in the United States. For instance, OA in the knees occurs in ~10% of males and 13% of females above the age of 60 (1). This number is likely to increase due to the aging population and the obesity epidemic (2). The most common modalities of treatment for OA include surgery and pharmaceutical intervention. For example, acetaminophen and non-steroidal anti-inflammatory drugs are commonly prescribed for pain relief in patients with OA. In more severe cases, steroid injections and surgical interventions are required (3).

Adipose tissue-derived stem cells (ADSCs) have been characterized as having the ability to self-renew and differentiate into different connective tissue cells, including osteoblasts, adipocytes, chondrocytes and myocytes, under specific inductive stimuli (4). ADSCs are abundant and can be easily acquired by liposuction with minimal donor morbidity (5). In total, 1-10% of nucleated cells in adipose tissue are ADSCs, whereas only 0.0001-0.01% of nucleated cells in bone marrow are stem cells (5). A previous study suggested that the age of the donor does not have a significant role in the phenotype or function of ADSCs (4). Most clinical trials which utilize ADSCs for OA treatment have been based on the autologous cells from the stromal vascular fraction, as have most of the *in vivo* studies (6,7). Although the use of ADSCs for treating OA has been gaining attention clinically and experimentally, the underlying mechanisms by which ADSCs attenuate OA have not been fully elucidated.

Exosomes are small, membrane-bound extracellular vesicles that have been shown to serve a role in intercellular communications; they are derived from the cell membrane during endocytic internalization. Exosomes are present and stable in

the blood and in synovial fluids (7). Emerging evidence shows that exosomes are involved in the development of joint diseases, such as OA and rheumatoid arthritis (8). The dysregulation of exosome secretion and/or uptake can lead to acute and chronic inflammation, followed by the degeneration of cartilage and the destruction of joints (9). Exosomes in the blood have been shown to possess both diagnostic and therapeutic values for joint disorders, such as OA (10-12). In the present study, the function and the mechanisms of exosomes released from ADSCs (ADSC-Exos) were investigated, in order to assess their therapeutic potential in the treatment of OA. ADSCs were isolated from an obese patient diagnosed with OA in order to establish a source of exosomes for further experiments. It was found that ADSC-Exos effectively promoted chondrocyte proliferation and migration. Furthermore, ADSC-Exos prevented the H_2O_2-induced apoptosis of chondrocytes and suppressed inflammatory markers in activated synovial fibroblasts (SFs). Mechanistically, it was demonstrated that ADSC-Exo treatment led to increased levels of chondrogenic microRNA (miR)-145 and miR-221, as well as chondrogenic markers, in periosteal cells. The present study provided evidence and a mechanistic explanation for the therapeutic applications of ADSC-derived exosomes in the treatment of OA.

Materials and methods

Isolation and characterization of ADSCs. The present study was conducted in compliance with the Declaration of Helsinki. The clinical specimens were obtained between July and October 2017 in Zhejiang Provincial People' Hospital, People's Hospital of Hangzhou Medical College. Informed written consent from all the participants was obtained. ADSCs were collected and isolated from adipose tissue during elective liposuction surgery of a healthy donor. The activated SFs were isolated from an obese patient diagnosed with OA in middle-aged male subjects (55-70 yrs). Adipose tissue (~1.5 g) was harvested from the subcutaneous adipose tissue and washed with PBS. The tissue was cut into strips and digested with collagenase (final concentration 1 mg/ml in 25 ml PBS) at 37˚C for 45 min, after which 25 ml of DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) was added to neutralize collagenase activity. The digested tissues were then filtered with a 0.22 μm filter and centrifuged at 800 x g for 6 min at 25˚C and the supernatant was discarded. The resulting pellet contained ADSCs. ADSCs were seeded at $5x10^4$ cells/cm^2 in 60 cm^2 tissue culture dishes and cultured with DMEM containing 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.), 100 units/ml penicillin and 100 μg/ml streptomycin. After 1 week of culture, the cells were harvested with 0.25% trypsin-EDTA, centrifuged 800 x g at 25˚C for 6 min n and washed twice with PBS. The ADSCs were then used for co-culture assays. To evaluate the multipotent potential of the ADSCs, the ADSCs were cultured under inductive conditions, where osteogenesis and chondrogenesis were promoted, according to an established protocol (13). The cell lineage and differentiation state were evaluated using immunohistochemical staining and quantitative PCR reactions.

Isolation and culture of primary synovial fibroblasts and periosteal cells. The isolation and culture of synovial tissues was performed using a previously established method (14). In brief, synovial tissues were minced, digested for 30 min at 37˚C in PBS containing 0.1% trypsin, followed by digestion with 0.1% collagenase in DMEM with 10% FBS for 1 h. The suspension was filtered and centrifuged 800 x g at 25˚C for 10 min. The pellet collected contained the cells of interest. The cells were cultured for 7 days in DMEM supplemented with 10% FBS, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B (Gibco; Thermo Fisher Scientific, Inc.). The non-adherent cells were carefully removed. Subsequently, the cells were harvested, resuspended in DMEM/Ham's F12 medium containing 10% human allogenic serum, plated in cell culture dishes (diameter = 15 cm), and allowed to attach for about 4-6 days. Moreover, primary periosteal cells were isolated from four independent donors. In brief, the periosteal flap was rinsed with Hanks solution (Biochrom) three times, minced and digested for 3 h in Dulbecco's modified eagle medium (DMEM)/Ham's F12 medium (Biochrom) containing 10,000 U/ml collagenase II (Biochrom), 10% human allogenic serum (German Red Cross), 2.5% Hepes (Biochrom) and 1% penicillin/streptomycin solution (Biochrom). Subsequently, the cells were harvested, resuspended in DMEM/Ham'sF12 medium containing 10% human allogenic serum, plated in cell culture dishes (diameter = 15 cm), and allowed to attach for about 4-6 days. Phenotype analysis of the expression of synovial fibroblasts markers, as well as that of synovial fibroblasts features previously reported at a tissue level, was conducted by flow cytometry in synovial fibroblasts, either negatively isolated from primary culture or obtained from conventional fourth passage.

Isolation of mesenchymal stem cells (MSCs) from adipose tissues. Briefly, adipose tissues were digested by collagenase using the EpiQuik Whole Cell Extraction kit (Invitrogen; Thermo Fisher Scientific, Inc.). The stromal vascular fractions (SVFs) were then resuspended into MSCs medium (Invitrogen; Thermo Fisher Scientific, Inc.). These cells were then sub-cultured to the 5th passage and used for experiments.

Exosome isolation and characterization. For exosome isolation, ADSCs between passage 4 and 8 were expanded in traditional 2D adherent cell culture and then seeded on BioNOC II micro carriers (Sigma-Aldrich, Merck KGaA). For seeding, $8x10^6$ cells resuspended in 50 ml of DMEM with 10% FBS, 1% L-glutamine and 1% P/S and layered on top of 2 g of sterilized BioNOC II in a 250 ml Erlenmeyer flask (CLS431144, Sigma-Aldrich, Merck KGaA). The cells were maintained in static incubation during the first 16 h and were then supplemented with additional 200 ml of medium. The supernatant was ultracentrifuged using a W32Ti rotor (L-80XP; Beckman Coulter, Inc.) at 110,000 x g for 70 min to pellet the exosomes. The pellet was washed in PBS and centrifuged for a second time at 110,000 x g for 70 min. The PBS was removed and the exosomes were re-suspended in 100 μl nuclease-free water. All centrifugation key steps were performed at 4˚C.

Measurement of TNF-α and IL-10 levels. Adipose-derived stem cell-derived Exos suppress inflammatory markers in activated SFs. The concentration of TNF-α and IL-10 levels

in chondrocytes was detected with an ELISA kit (DTA00C and D1000B, R&D Systems) according to the manufacturer's instructions.

Induction of apoptosis. Patient-derived articular chondrocytes were isolated and seeded at a density of the 5,000 cells/well in 12-well plate and cultured for 5-7 days until confluency was reached. Chondrocytes were exposed to 100 μM H_2O_2 with or without exosomes (1, 5 and 10x10^{10} particles/ml) for 1 h at 37˚C. The resultant cells were then subjected to further analysis. The effect of chondrocytes and exosomes on cell apoptosis was determined using an Annexin V-FITC Apoptosis Detection kit [cat. no. 1035-100, Hangzhou Multisciences (Lianke) Biotech Co., Ltd.] and flow cytometry. Annexin V-FITC binding and PI staining [cat. no. 1035-100, Hangzhou Multisciences (Lianke) Biotech Co., Ltd.] were detected using a flow cytometer and analyzed with FACSDiva v8.0.3 (Becton-Dickinson) software. The experiments were performed independently three times.

Reverse transcription-quantitative (RT-q)PCR. RNA samples were extracted and quantified using the Qubit HS RNA kit (cat. no. 2019-0023, Thermo Fisher Scientific, Inc.) with a Qubit 3.0 Fluorometer (v2.0.3 software, Thermo Fisher Scientific, Inc.), following the manufacturer's instructions. The synthesis of complementary DNA was performed using the PrimeScript™ RT reagent kit [cat. no. 2312, Hangzhou Multisciences (Lianke) Biotech Co., Ltd.]. RT-qPCR was performed using a human miRNA RT-qPCR detection kit specifically for *Homo sapiens* (hsa)-miR-145, hsa-miR-221, hsa-miR-194, hsa-miR-101 and RNA U6 small nuclear 6 pseudo-genes (RNU6B; cat. no. 33456, Guangzhou RiboBio Co., Ltd.). RNU6B was used for normalization of miRNA expression levels. qPCR was performed using SYBR® and GAPDH was used for normalization of the results. The sequences of target gene were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and miRBase (http://www.mirbase.org/). The primers were designed using Primer Designer 2.0, and the sequences are shown below. The miR-specific primers were: hsa-miR-145 AGCGAGTGCAGT GGTGAAA, hsa-miR-221 GAGTGGGGGTGGGACATAAA, hsa-miR-3188 GTGCGGATACGGGGAAAA, hsa-miR-194 TGGGCTGGGTTGGGAAA and hsa-miR-101 GGCCCA GTGGGGGGAA. The PCR conditions: 95˚C pre-denaturation for 15 min, followed by 40 cycles of denaturation at 94˚C for 15 sec, annealing at 55˚C for 30 sec and extension at 70˚C for 30 sec. Relative gene expression was analyzed using the $2^{-\Delta\Delta Cq}$ method. U6 small nuclear RNA was used as a miRNA internal control. miRNA and U6 (cat. no. HmiRQP9001) primers were purchased from iGeneBio (GeneCopoeia, Inc.). All primers were synthesized by Beijing Liu He Synthetic Genomics Ltd. U6 was used as the internal control.

Western blotting. All western blotting analyses were performed according to established protocols (13). The following primary antibodies were obtained from Cell Signaling Technology, Inc.: Interleukin (IL)-6 (cat. no. 8904, dilution, 1:1,000), nuclear factor-κB (NF-κB) (cat. no. D14E12, dilution, 1:2,000), tumor necrosis factor-α (TNF-α) (cat. no. D2D4, dilution, 1:1,500), IL-10 (cat. no. D13A11, dilution, 1:2,000) and β-actin (cat. no. 12E5, dilution, 1:5,000). The transcription factor SRY-box transcription factor 9 (Sox9) primary antibody was obtained from Abcam (cat. no. D8G8H, dilution, 1:1,000). Collagen type II (Col II cat. no. E819H, Abcam, dilution, 1:1,000) was analyzed using 5% SDS-PAGE gels with an anti-Col II primary antibody (cat. no. E819H, Abcam, dilution, 1:1,000). Next, the membrane was washed and incubated with goat anti-rabbit secondary antibody (cat. no. 33456, Abcam, dilution 1:5,000). Finally, an enhanced chemiluminescence (ECL, Biovision) kit was used to observe protein bands in a ChemiDoc XRS Plus luminescent image analyzer (Bio-Rad Laboratories, Inc.). β-actin was used as the internal control in the experiment, and therefore, the ratio of the integrated optical density of the targeted protein bands to that of β-actin in every group was determined as the final relative protein expression intensity. All experiments were replicated 3 times.

Alcian blue staining. Osteogenic differentiation was assessed using alcian blue staining. Briefly, tissue was embedded in paraffin and sections (4.0 μm thick) were mounted onto glass slides, cleared using xylene, and gradually rehydrated. Antigen retrieval was performed on slides using sodium citrate buffer (10 mM sodium citrate and pH 6) at 100˚C for 20 min. Slides were cooled and rinsed in distilled water to remove citrate solution. Slides were blocked using Super Block (Thermo Fisher Scientific, Inc.) for 1 h at room temperature. For slides stained with alcian blue staining, sections were stained with alcian blue solution (Sigma-Aldrich, Merck KGaA) and Weigert's Iron Hematoxylin (Sigma-Aldrich, Merck KGaA) according to the manufacturer's protocol. Alcian blue Stain kit (Connective Tissue Stain; Abcam) was also used to stain sections according to the manufacturer. For slides stained with hematoxylin and eosin (H&E), sections were cleared and rehydrated followed by staining with H&E, according to standard protocols. Sections were then dehydrated gradually and mounted for imaging. All images were acquired using a Nikon Digital Imaging System.

Von Kossa staining intensity analysis. After removing the culture medium, the cells were rinsed twice with PBS and fixed with 95% ethyl alcohol and 5% isopropyl alcohol for 2 h at 4˚C. Subsequently, the cells were rinsed twice with deionized water. The cells were treated with 2.5% silver nitrate solution in deionized water at room temperature for 24 h, rinsed twice with deionized water, and 1 ml freshly prepared 0.5% hydroquinone solution in deionized water was applied to the cell layers on microscope slides and incubated for 2 min in the dark at room temperature. The hydroquinone solution was removed and 1 ml freshly prepared 5% sodium thiosulfate solution in deionized water was added for 2 min at room temperature. The cells were washed again with deionized water. The staining was semi-quantitatively evaluated under a light microscope using the color intensity of the *Von Kossa* staining. Under a microscope, measurements were made in 10 consecutive visual areas at 400x magnification.

Proliferation of chondrocytes. The effect of exosome stimulation on chondrocytes was measured using the 5-ethynyl-2'-deoxyuridine (EdU)-488 Cell Proliferation kit (Guangzhou RiboBio Co. Ltd.) and flow cytometry, according to the manufacturer's instructions. Briefly, normal chondrocytes were seeded into 48-well plates and cultured

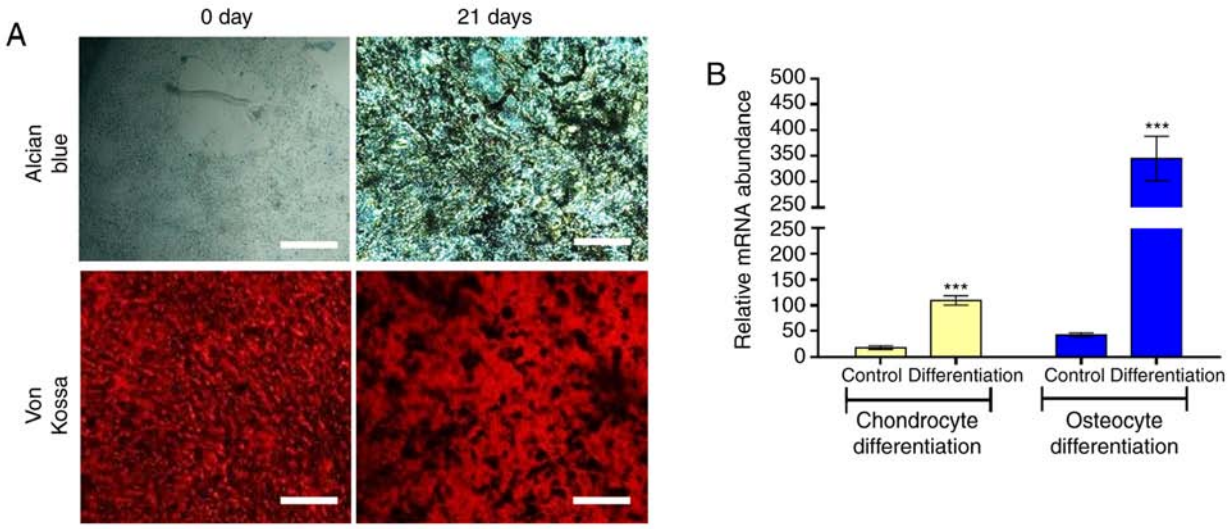

Figure 1. Characterization of ADSCs. (A) Differentiation assays for the isolated ADSCs. ADSCs were cultured under chondrogenic and osteogenic differentiation conditions. After 3 weeks of differentiation, alcian Blue and Von Kossa stains were used to determine the cell lineages (alcian blue, chondrocytes; Von Kossa, osteocytes). Scale bar, 10 μm. (B) Reverse transcription-quantitative PCR analysis for specific gene markers was used to identify cell lineages. The mRNA expression levels in the differentiated cells were significantly higher than in the undifferentiated counterparts (Col II, chondrocytes; Runx2, osteoblasts). ***P<0.001. ADSCs, adipose-derived stem cells; Col II, collagen type II; Runx2, runt-related transcription factor 2.

with exosomes (400 μg/ml) for 48 h. EdU working solution, consisting of 150 μl complete culture medium for chondrocytes supplemented with 0.15 μl EdU, was added per well and incubated at 37˚C for 3 h. The cells were then trypsinized, washed with PBS, fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature, neutralized with 2 mg/ml glycine for 2 h and washed again with PBS. Permeabilization was performed using 0.4% Triton X-100 for 5 min at room temperature and the cells were washed twice with PBS. The labelled chondrocytes were resuspended in the staining solution from the kit, incubated for 10 min at room temperature in the dark, washed twice with 0.4% Triton X-100 and then resuspended in PBS for flow cytometry analysis and analyzed with FACSDiva v8.0.3 software (Becton-Dickinson).

Migration of chondrocytes. A chondrocyte migration assay was performed using Transwell inserts. In total, $5x10^4$ chondrocytes were seeded into the upper chambers of the Transwell (8 μm pores) in 10% FBS chondrocyte culture DMEM media, while the lower chamber was filled with 600 μl chondrocyte culture medium containing exosomes (400 μg/ml). The chondrocytes were allowed to migrate at 37˚C for 12 h. The non-migrated chondrocytes remaining on the upper surface of the insert were carefully removed. The migrated chondrocytes on the bottom surface of the insert were fixed with 4% PFA for 15 min at room temperature and stained with 0.5% crystal violet for 10 min at room temperature, before washing with PBS. In total, five fields were randomly selected and photographed (magnification, x100) using a Leica light microscope (Leica Microsystems, Inc.).

Statistical analysis. Statistical analysis was performed with SPSS v11.0 (SPSS, Inc.). Data represents assays performed a minimum of three times in triplicates. Data are presented as the mean ± SD. Comparisons of macroscopic and histological scores were performed using the Mann-Whitney U test. Comparison made among multiple groups were performed using ANOVA and Tukey's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Characterization of ADSCs. The multipotency of ADSCs obtained from an obese patient with OA was assessed. ADSCs were cultured under different inductive conditions. For example, under osteogenic conditions, ADSCs differentiated along the osteogenic lineage, as shown by the increased expression of runt-related transcription factor 2 (Runx2) mRNA (~1,000-fold higher than the control) and the von Kossa staining intensity of deposited calcium phosphate. (Fig. 1A and B). The increased Col II mRNA (~250-fold higher than the control) and Alcian Blue staining was a method to detect the glycosaminoglycans in cells that were indicators of chondrogenic differentiation (Fig. 1A and B). These data provided *ex vivo* evidence that the primary ADSCs obtained in the present study were multipotent.

ADSC-derived exosomes (ADSC-Exos) decrease inflammation in activated SFs and macrophages. Secreted exosomes have been reported to be involved in communication among cells and shown to participate in a wide spectrum of cellular processes, including the pathological development and progression of arthritis (5,6). In the present study, secreted exosomes were isolated from ADSCs and incubated with activated SFs obtained from an obese patient with OA. The results demonstrated that exosome-treated SFs exhibited significantly reduced levels of inflammatory biomarkers, namely IL-6, TNF-α and NF-κB, while there were increased levels of IL-10 (Fig. 2A and B). The microenvironment was also assessed by examining the synovial macrophages. It was found that ADSC-Exos had immune-suppressive effects on the microphages; the M1 type macrophage marker TNF-α was

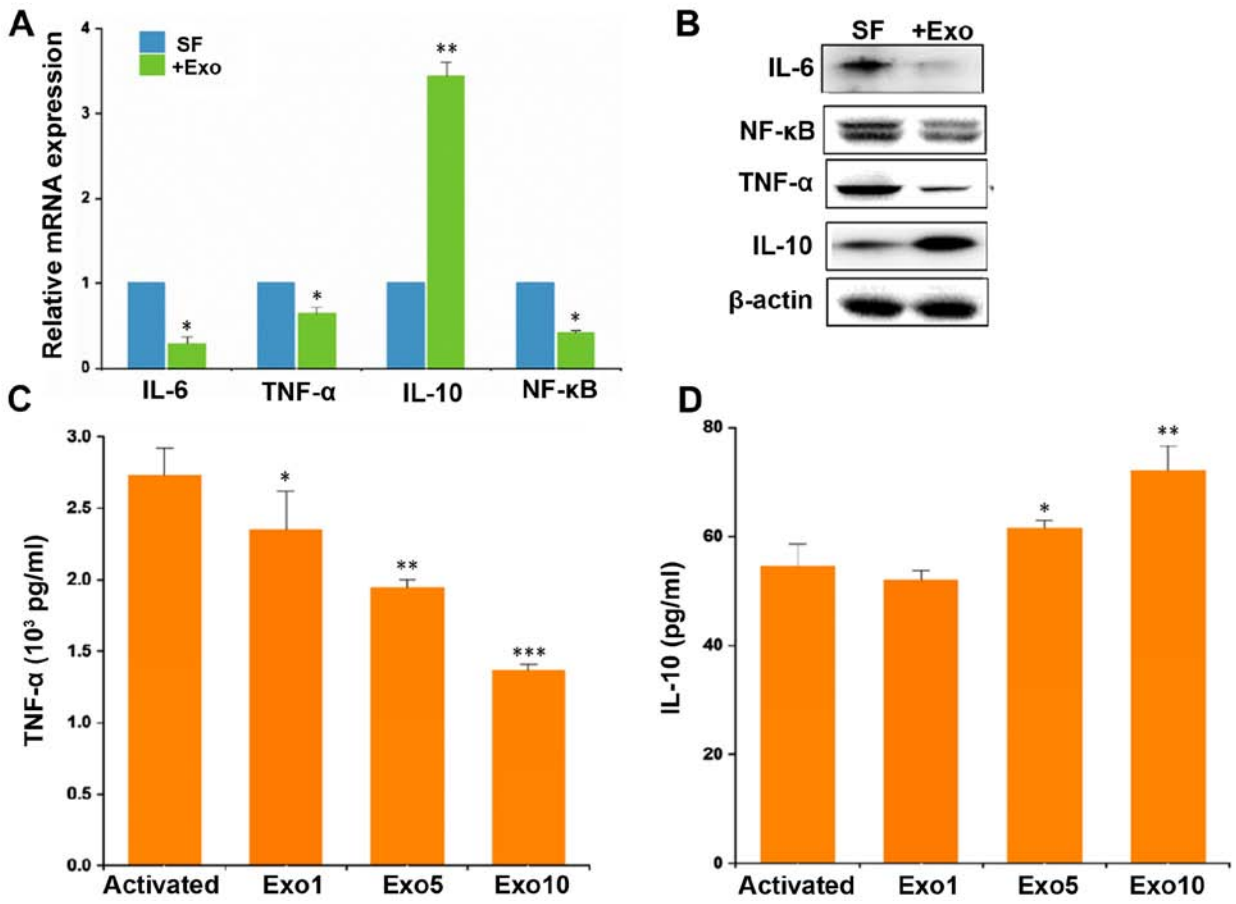

Figure 2. Adipose-derived stem cell-derived Exos suppress inflammatory markers in activated SFs. (A) RT-qPCR analysis of Exo-treated activated SFs obtained from a patient with osteoarthritis. (B) Western blotting of activated SFs treated with or without exosomes. (C) Synovial macrophages were treated with Exos (Exo1, $1x10^5$ particles/ml; Exo5, $5x10^5$ particles/ml; Exo10, $10x10^5$ particles/ml) and TNF-α and (D) IL-10 levels were evaluated. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ vs. untreated. Exo, exosome; SF, synovial fibroblast; RT-qPCR, reverse transcription-quantitative PCR; TNF-α, tumour necrosis factor-α; IL, interleukin.

significantly downregulated by treatment with ~10^{10} exosomes particles/ml, while IL-10 was upregulated (Fig. 2C and D).

ADSC-Exos protect articular chondrocytes and promote mesenchymal differentiation and migration. After establishing that exosomes from ADSCs reduced inflammation in SFs, their actions on articular chondrocytes were investigated. Primary articular chondrocytes were cultured and subjected to H_2O_2 insults in order to mimic the oxidative stress induced by M1 macrophages in the arthritic synovium (9,10). H_2O_2 treatment induced apoptosis in the articular chondrocytes. When a sufficient number of exosomes were added to the articular chondrocytes treated with H_2O_2, significantly less apoptotic chondrocytes were detected (Fig. 3A). For example, ~37% of chondrocytes treated with exosomes (Exo 10 group; $10x10^{10}$ particles/ml) survived H_2O_2 treatment (Fig. 3A). In addition, the effects of ADSC-derived exosomes on the migration of MSCs was assessed. The migratory ability of MSCs appeared to increase in a dose-dependent manner with increasing exosome treatment (Fig. 3B). The morphogenic effects of ADSC-Exos on MSCs were then investigated. It was found that MSCs incubated with exosomes had a higher differentiation potential, including osteogenesis, adipogenesis and chondrogenesis. This was reflected by the increased mRNA expression of peroxisome proliferator-activated receptor-γ (PPARγ; a transcription factor involved in adipocyte cells differentiation), Col II (a marker of chondrocyte differentiation) and Runx2 (an osteogenesis marker). Runx2 was the most elevated in the MSCs (~1,000-fold higher) following incubation with exosomes (Fig. 3C).

ADSC-Exos promote chondrogenesis in vitro. As ADSC-Exos induced Runx2 mRNA expression in MSCs, the chondrogenesis-promoting effect of exosomes on periosteal cells (precursor cells) was investigated. ADSC-Exo incubation led to increased Alcian Blue staining in a time-dependent manner (Fig. 4A). Periosteal cells exhibited chondrocyte morphology, with a substantial Alcian Blue staining intensity, after 10 days of incubation (Fig. 4A). The increased Alcian Blue staining was accompanied by the increased mRNA expression of chondrogenesis markers, namely Sox9, Col II and β-catenin (Fig. 4b). The increased β-catenin mRNA in the exosome-treated periosteal cells was consistent with previous studies that indicated that the Wnt/β-catenin/T cell factor-mediated transcription process served an important role in chondrocytic differentiation and proliferation (11,12). To test this hypothesis, ICG-001, an antagonist of Wnt/β-catenin signaling, was introduced along with the exosome treatment. The presence of ICG-001 appeared to completely prevent the periosteal cells from differentiating into chondrocytes, as reflected by the absence of Alcian Blue stain (Fig. 4C). Consistently, the RT-qPCR analysis of the ICG-001+exosome-treated periosteal cells

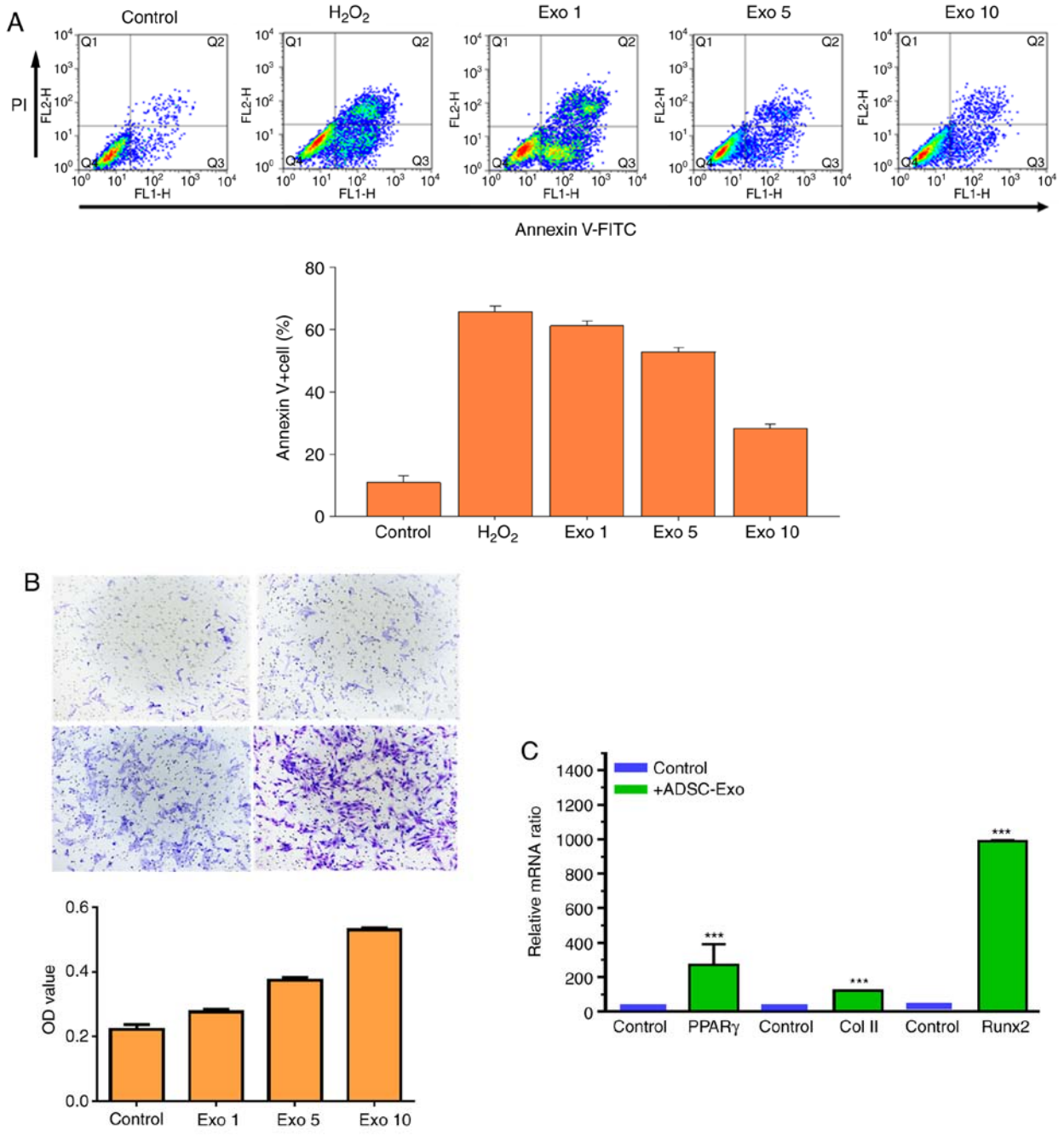

Figure 3. ADSC-derived Exos suppress apoptosis and promote migration and differentiation of MSCs. (A) Articular chondrocytes were subjected to H_2O_2 treatment to simulate oxidative stress in the arthritic microenvironment. Different doses of Exos (Exo1, $1x10^5$ particles/ml; Exo5, $5x10^5$ particles/ml; Exo10, $10x10^5$ particles/ml) were used to investigate their effect against H_2O_2-induced oxidative stress and apoptosis. The upper panel shows representative flow cytometry plots of Annexin V-FITC/PI labeling; the lower panel shows the quantification of Annexin V-positive cells. The control group represents the baseline apoptotic status of the articular chondrocytes without H_2O_2 treatment. (B) Transwell chamber migration assay. Exo treatment increased the migratory ability of MSCs in a dose-dependent manner. The upper panels show representative images of the inserts and the lower panels show representative images of the migrated MSCs. Quantification is shown from three independent repeats. (C) ADSC-derived Exos increased the mRNA expression levels of PPARγ, Col II and Runx2. The data is represented as a ratio to the control group (set as 1). ***P<0.001 vs. untreated. ADSC, adipose-derived stem cell; Exo, exosome; MSCs, mesenchymal stem cells; PI, propidium iodide; PPAR-γ, peroxisome proliferator-activated receptor-γ; Col II, collagen type 2; Runx2, runt-related transcription factor 2.

revealed that the expression of markers of chondrogenesis (Sox9, Col II and β-catenin) was effectively reversed (Fig. 4D).

ADSC-derived exosomes promote chondrogenesis by upregulating miR-145 and miR-221. After observing that the ADSC-derived exosomes induced the expression of pro-chondrogenic markers, the underlying mechanism of action was investigated. Several established miRNAs responsible for promoting chondrocyte differentiation and proliferation were screened. Among these miRNAs, miR-145 and miR-221 were increased following ADSC-Exo treatment, while no significant changes were identified in the levels of miR-101 and miR-194 (Fig. 5A). The effects of miR-221 and miR-145 on periosteal cells were examined using transient transfections of their

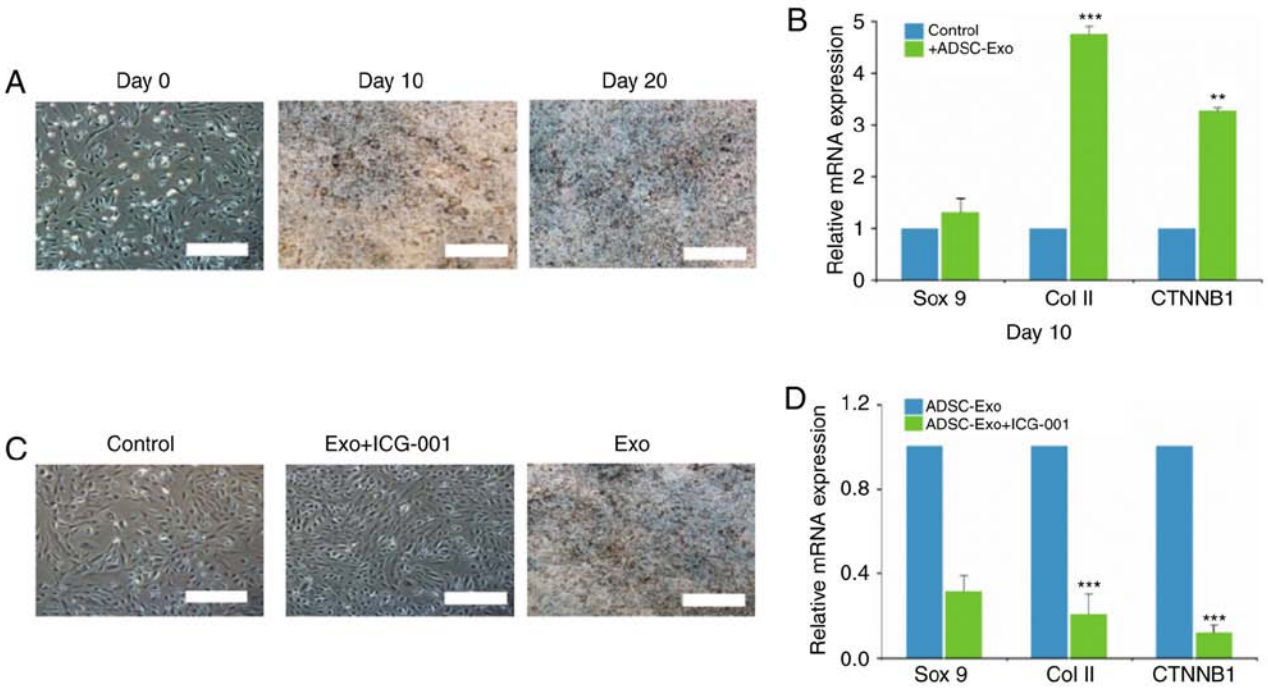

Figure 4. ADSC-derived Exos induce chondrogenesis in periosteal cells. (A) Periosteal cells cultured with ADSC-derived Exos ($10x10^{10}$ particles/ml) exhibited increased Alcian Blue staining over time, indicative of chondrocytic differentiation. (B) RT-qPCR analysis of Sox9, Col II and CTNNB1 expression in periosteal cells treated with Exos. (C) The addition of ICG-001, a Wnt/β-catenin antagonist, abolished the chondrogenic effects of ADSC-derived Exos, as evidenced by Alcian Blue staining. (D) RT-qPCR analysis of Sox9, Col II and CTNNB1 expression in periosteal cells cultured with ADSC-derived Exos, in the presence or absence of ICG-001. **P<0.01, ***P<0.001. ADSC, adipose-derived stem cell; Exo, exosome; RT-qPCR, reverse transcription-quantitative PCR; Sox9, SRY-box transcription factor 9; Col II, collagen type 2; CTNNB1, β-catenin.

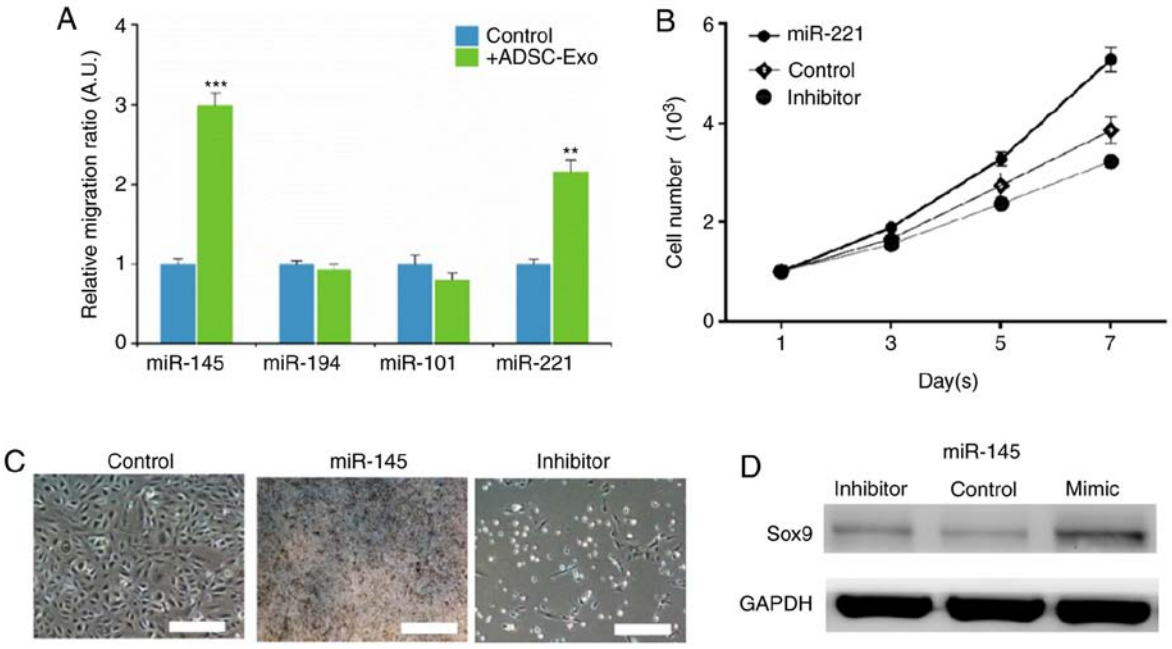

Figure 5. ADSC-derived Exos regulate miRNA expression in chondrogenesis. (A) Reverse transcription-quantitative PCR analysis of chondrogenesis-associated miRNAs. Exo-treated periosteal cells exhibited a significant increase in the expression levels of miR-145 and miR-221, while no changes were observed in the levels of miR-101 and miR-194. **P<0.01; ***P<0.001 vs. control. (B) miR-221 mimic transfection promoted proliferation in periosteal cells, while a miR-221 inhibitor reduced proliferation compared with the scramble control. (C) miR-145 mimic transfection significantly increased collagen deposits, as observed by Alcian Blue staining, while a miR-145 inhibitor transfection resulted in little or no Alcian Blue staining. (D) Western blotting for Sox9 protein expression levels in periosteal cells transfected with the miR-145 mimic or inhibitor. ADSC, adipose-derived stem cell; Exo, exosome; miR/miRNA, microRNA; Sox9, SRY-box transcription factor 9.

respective mimics and inhibitors into periosteal cells (Fig. S1). The mimic-transfected periosteal cells had increased viability, while the opposite was found in the cells transfected with the miR-221 inhibitor (Fig. 5B). In addition, periosteal cells

transfected with the miR-145 mimic had a marked increase in alcian Blue staining compared with almost no staining in the inhibitor-transfected cells (Fig. 5C); this result was supported by western blotting, which showed that the expression of Sox9 was reduced in the inhibitor-transfected periosteal cells compared with the mimic-transfected cells (Fig. 5D).

Discussion

OA is a common joint disorder that affects millions of patients globally (1). Inflammation has been shown to be a major underlying mechanism of OA, which not only initiates but also propagates the degradation of the chondrocytes in the joints of patients with OA (14). In the present study, the potential of exosomes isolated from ADCS in reducing inflammation and promoting chondrogenesis was investigated *in vitro*. The present findings suggested that ADSC-Exos may be beneficial as a therapeutic tool in patients with OA.

Adipose tissue contains a subset of cells with the ability to differentiate into multiple lineages. ADSCs have been demonstrated to differentiate into multiple different cell types, including adipocytes, osteoblasts, chondrocytes and myocytes, in specific inductive conditions (15). Due to the multipotent nature of ADSCs, researchers and clinicians have been exploring their therapeutic potential in various joint disorders, including OA (16). Another advantage of ADSCs is the avoidance of rejection. Autologous ADSCs can now be easily isolated in substantial quantities from subcutaneous adipose tissues and cultured *ex vivo* (17). In addition, ADSCs can be harvested in a much less invasive manner compared with bone marrow-derived MSCs (4). Previous studies have shed light on the underlying mechanism of stem cell proliferation and communication within the stem cell niche. One such mechanism involves the secretion and uptake of exosomes (18). Exosomes have been shown to participate in stem cell- or progenitor cell-mediated tissue regeneration. In the bones, exosomes regulate multiple signaling processes involved in the differentiation of osteocytes and bone architecture maintenance in a paracrine manner (6). For instance, exosomes were detected in the bone microenvironment, where they modulated intracellular or intercellular signaling, by targeting the same cells, adjacent cells or distant organs (16). Exosomes have been considered to be an important component in the delivery of chondrogenic signaling factors for bone regeneration (19).

The present study demonstrated that exosomes isolated from ADSCs activated SFs and downregulated the pro-inflammatory markers IL-6 and NF-κB; without exosomes, SFs had significantly higher expression levels of IL-6. Previous studies indicated that the synovial inflammation and cartilage destruction characteristic of patients with OA were largely influenced by IL-6 and NF-κB (1,2); therefore, the ability of ADSC-Exos to suppress these pro-inflammatory cytokines suggested they have the potential to ameliorate OA. Treatment with exosomes suppressed apoptosis and promoted chondrogenesis in periosteal cells. Several miRNAs responsible for promoting chondrocyte differentiation and proliferation were screened. Among these miRNAs, miR-145 and miR-221 were elevated after exosome treatment, while no significant changes were identified in the levels of miR-101 and miR-194. Previous studies indicated that exosomes contain regulatory molecules, including RNAs, peptides, miRNAs and proteins, all of which participate in altering cellular signaling (20,21). One such example is the modulation of inflammation. Either intrinsic or exogenous exosomes have been shown to modulate inflammation and tissue repair in OA (21). In agreement with this, miR-145 has been implicated in several previous studies to have a role in suppressing TNF-α mediated inflammation and attenuating the degradation of cartilage (22). The reduced level of miR-221 has been associated with osteoporosis and cartilage degradation (23); the increased expression of miR-221 in exosome-treated periosteal cells promoted the proliferation of periosteal cells, which have the potential to differentiate along the chondrogenic or osteogenic lineages (24).

In conclusion, the present study demonstrated that ADSC-Exos may have a therapeutic value. ADSC-Exos exerted a strong stimulatory effect on chondrocyte migration and proliferation. As ADSCs can be obtained in a patient-specific manner and are theoretically inexhaustible, ADSC-Exos may represent a novel therapeutic approach for the treatment of OA in future clinical settings.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

CZ was responsible for the conception and design of the study. WP and BY performed the experiments and analyzed and interpreted the data. QB was responsible for the collection and assembly of data. JC and YX performed data analysis and interpretation. The final version of the manuscript has been read and approved by all authors.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Zhejiang Provincial People's Hospital, and informed consents were obtained from the patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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