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Original Article

Allogeneic adipose-derived mesenchymal stem cells promote the expression of chondrocyte redifferentiation markers and retard the progression of knee osteoarthritis in rabbits

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Abstract: Osteoarthritis (OA) is a progressively degenerative disease of joints. *In vitro* culture of chondrocytes results in dedifferentiation, which is characterized by the development of fibroblast phenotypes, reduced ability to produce cartilage extracellular matrix (ECM) and increase the expression of molecular markers Col1a1, Col10a1 and Runx2. Redifferentiation of chondrocytes is indicated by increased expression of the molecular markers Col2a1, Aggrecan and Sox9. In the current study, we investigated the effects of allogeneic rabbit adipose-derived mesenchymal stem cells (ADSCs) on articular chondrocytes, and explored the therapeutic effect of ADSCs on damaged articular cartilage at different stages in a rabbit OA model. *In vitro*, the proliferation and migration of primary articular chondrocytes were enhanced by cocultured rabbit ADSCs, and the expression of redifferentiation markers in chondrocytes cocultured with ADSCs was increased at both the mRNA and protein levels, while the expression of dedifferentiation markers was decreased. *In vivo*, the rabbit model of OA was established by anterior cruciate ligament transection (ACLT) with complete medial meniscectomy (MMx). Two weeks after surgery, ADSCs were used for OA rabbit treatment. Intra-articular injection of ADSCs gradually alleviated articular cartilage destruction, decreased Osteoarthritis Research Society International (OARSI) and Mankin scores, and reduced MMP13 expression at different stages in the rabbit model of OA. During the experiment, allogeneic ADSCs did not cause any adverse events. The current study demonstrates the effects and molecular mechanisms of ADSCs on articular chondrocytes and provides a favorable reference for clinical OA treatment with mesenchymal stem cells (MSCs) derived from adipose tissue.

Keywords: ADSCs, osteoarthritis, articular chondrocyte, molecular marker, articular cartilage

Introduction

Osteoarthritis (OA) is a progressively degenerative disease characterized by articular cartilage degradation, subchondral bone deterioration, and ultimately, by joint destruction [1, 2]. Articular cartilage covers the ends of bones of the diarthrodial human joints and is composed of highly specialized chondrocytes, sparsely surrounded by the dense extracellular matrix (ECM) [3]. Due to its avascular characteristics, articular cartilage has limited ability to respond to external and internal injury [4]. In recent years, the prevalence of OA has risen with an aging and increasingly obese population. The latest statistical data demonstrate that OA affects 250 million people aged over 60 years globally, and is associated with substantial morbidity, involving disability and reduced quality of life, which ultimately contributes to mortality [5, 6]. Therefore, this disease represents a significant economic burden for individuals and society. Conventional treatments include exercise, physiotherapy, lifestyle changes and analgesic medication. These approaches achieve temporary amelioration of the symptoms but do not alleviate or reverse the OA process. Although joint replacement is effective, the artificial joint has a limited lifespan and is unsuitable for younger patients with early-onset

OA who have an active lifestyle [7]. Therefore, novel therapeutic strategies that target the fundamental processes of OA are urgently required.

For over a decade, autologous chondrocyte implantation (ACI) has been used successfully for cartilage regeneration, and initial ACI clinical trials have yielded promising results [8]. However, ACI requires the removal of healthy cartilage tissue from the patient. Furthermore, the application of this technique is greatly limited by the low proliferative capacity of chondrocytes and the poor quality of the synthesized ECM [9]. Recently, mesenchymal stem cells (MSCs) have emerged as a promising alternative cell type to circumvent some of the disadvantages of ACI in the treatment of OA. MSCs, which can be isolated from many adult organs, possess self-renewal capability, and can differentiate into multiple lineages including adipocytes, osteoblasts and chondrocytes under certain conditions [10]. MSCs isolated from bone marrow (BMSCs), especially autologous BMSCs, have been widely used in animal models and clinical cases of OA treatment. Adipose-derived MSCs (ADSCs) represent a more readily available source of stem cells, and are more easily cultured and grow more rapidly *in vitro* [11]. Recently, ADSCs have attracted increasing attention and have been used in animal experiments and some clinical cases. Nevertheless, the results of clinical application are not always consistent and require further investigation in preclinical and clinical studies [12, 13]. It has been reported that intra-articular injection of allogeneic ADSCs was safe in rabbits and other species [14-19]. We performed this preclinical study to investigate the effects and mechanisms of allogeneic rabbit ADSCs on articular chondrocytes *in vitro* and articular cartilage *in vivo*.

Methods

Isolation and culture of rabbit ADSCs

All adult New Zealand white rabbits were purchased from the experimental animal center of Xi'an Jiaotong University. A rabbit weighing approximately 2 kg was anesthetized with 3% (40 mg/kg) pentobarbital sodium injected intravenously along the ear margin under sterile conditions. Adipose tissue was taken from subcutaneous fat pads of the neck and back, carefully minced and transferred to 0.1% type I collagenase (Sigma, MO, USA). After incubation with shaking at 37°C for 2 h, the digested solution was centrifuged at 1,000×*g* for 10 min to collect the cells. After washing twice with PBS, cells were cultured in F12/DMEM (HyClone, UT, USA) containing 10% FBS (Gibco, NY, USA) and 1% penicillin/streptomycin (Thermo, MA, USA), and maintained at 37°C in a humidified incubator under 5% CO_2.

Multilineage differentiation of ADSCs

Passage 3 ADSCs were seeded into 6-well plates for induction of adipogenic, osteogenic and chondrogenic differentiation [20]. After 14 days, cells were fixed with 4% paraformaldehyde (Boster, Beijing, China) for 30 min and stained with oil red O for identification of differentiated adipocytes, Alizarin red for differentiated osteocytes and toluidine blue (Solarbio, Beijing, China) for differentiated chondrocytes. Images were captured under an inverted microscope (Leica, Heidelberger, Germany).

Dual-immunofluorescent staining of ADSCs

ADSCs seeded on coverslips were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 8 min and blocked with 4% normal goat serum in PBS buffer for 1 h. The cells were then incubated with primary detection antibodies against CD44 and CD90 (Abcam, Cambridge, UK, 1:1000) at 4°C overnight. Subsequently, cells were incubated with anti-mouse FITC-conjugated and anti-rabbit CY3-conjugated secondary antibodies (Boster, Beijing, China, 1:200) at room temperature for 2 h. The cells were then labeled with DAPI for 3 min and images were acquired under a fluorescence microscope.

Isolation and characterization of rabbit primary chondrocytes

Under sterile conditions, the surface layer of the knee articular cartilage was carefully removed, cut into pieces, digested with 0.25% trypsin (HyClone, UT, USA) for 20 min followed by digestion with 0.2% type II collagenase at 37°C with shaking for 3 h. A 100-μm filter was then used to remove debris. After centrifugation at 1,000×*g* for 10 min, the supernatant was removed, and the articular chondrocytes were washed twice with PBS. Subsequently,

Table 1. The primer information for quantitative PCR

Gene name	Aligned mRNA no.	Sequence (5'-3')	Size (bp)	Annealing temperature (°C)
Aggrecan	L38480	For: GTCAGGTACCCCATCCACAC	156	64
		Rev: CGACGTTGCGTAAAAGACCT		
Col2a1	NM_001195671	For: GGAATCCCAGGACCCATC	135	58
		Rev: AAAGGCGcACATGTCGAT		
SOX9	AY598935	For: AGAACAAGCCCCACGTCA	219	58
		Rev: TGTAGTCCGGGTGGTCTTTC		
Col1a1	XM_017348831.1	For: GCTCCCCAGATGTCTTACGG	219	60
		Rev: TCCGTCATCTCCGTTCTTGC		
Col10a1	XM_002714724.3	For: GCCCTTCTGCTGCTAGTGTCTT	108	62
		Rev: CTGTGTCTTGGTGTTGGGTTGT		
Runx2	XM_017345159.1	For: CCGTCCATTCACTCCACCAC	119	60
		Rev: GAAGACTGGGAGTCCAAGGTG		
GAPDH	NM_001082253	For: CAACATCAAGTGGGGTGATG	201	62
		Rev: GCGTTGCTGACAATCTTGAG		

the cells were maintained in the same medium used to culture ADSCs. Chondrocytes were characterized by toluidine blue staining and immunofluorescent labeling of Col2a1.

Proliferation assay

Primary chondrocytes were seeded in the lower chamber of 24-well transwell plates (5×10^4 cells/well) containing a porous insert (0.4 μm pore size) and cocultured with ADSCs (2×10^5 cells/well) seeded in the upper chamber. After incubation for 24 h at 37°C, chondrocyte proliferation was determined using an EdU assay kit (Solarbio, Beijing, China) according to the manufacturer's instructions.

Migration assay

Primary chondrocytes (3×10^4 cells/well) were seeded in the upper chamber of 24-well transwell plates containing a porous insert (8 μm pore size) and cocultured with ADSCs (1×10^5 cells/well) seeded in the lower chamber. After incubation for 24 h at 37°C, the inserts were fixed with 4% paraformaldehyde for 20 min and stained with 0.5% crystal violet solution for 15 min. The upper surface of the insert membrane was carefully wiped with a cotton swab to remove unmigrated cells [21].

RNA extraction and real-time quantitative PCR (RT-qPCR)

Chondrocytes were seeded in the lower chamber of 6-well transwell plates (1×10^5 cells/well) containing a porous insert (0.4 μm pore size) and cocultured with ADSCs (2×10^5 cells/well) seeded in the upper chamber. After 24 h, total RNA was extracted and reverse transcribed to first strand cDNA as described previously [22]. Expression of the target genes and the internal control gene GADPH was quantified with FastStart Universal SYBR Green Master (Rox, CO, USA) using the primers shown in **Table 1**.

Protein extraction and western blot analysis

Chondrocytes were cocultured with ADSCs in 6-well transwell plates as mentioned. Total proteins were extracted, quantified, separated and transferred to polyvinylidene difluoride (PVDF) membranes as described previously [22]. The membranes were incubated overnight at 4°C with primary detection antibodies against Sox9 (CST, MA, USA, 1:1000), Col2a1 (Bioss, Wuhan, China, 1:200), Aggrecan (Abways, Shanghai, 1:400), Col1a1 (Bioss, Beijing, China, 1:500), Col10a1 (Bioss, Beijing, China, 1:500), Runx2 (CST, MA, USA, 1:1000) and GADPH (ZSGB-BIO, Beijing, China, 1:1000). GAPDH was used as internal control on the same membrane. Membranes were then incubated with anti-rabbit IgG secondary detection antibody (Thermo, MA, USA, 1:5000) at room temperature for 2 h. The signal intensity of target proteins was determined using an enhanced chemiluminescence system (Millipore, MA, USA). The results were scanned using the GeneGnome XRQ System, and the area and density of specific western blot bands were analyzed with the GeneTools analysis software (Syngene, MD, USA).

Rabbit knee OA model induction

The rabbit OA model was established by anterior cruciate ligament transection (ACLT) with complete medial meniscectomy (MMx). Twenty four male adult New Zealand white rabbits were subjected to the surgery for subsequent experiments. Under sterile conditions and general anesthesia, the rabbit was placed in the supine position on the operating table. A longitudinal incision of approximately 4 cm was then made in the medial patella using an 8-10 mm medial parapatellar approach. The patella was pushed outward to dislocate the knee joint. With the joint in the maximum flexion state, the anterior cruciate ligament was transected and the medial meniscus was completely resected in both hind legs using ophthalmology scissors. The junction of the meniscus and medial collateral ligament remained intact and the articular cartilage surface was not damaged [23, 24]. After a positive drawer test, the wound was sutured layer by layer and fixed with a sterile dressing. Each rabbit was injected with penicillin (20,000 U/mL) every day for three consecutive days and allowed to move, eat and drink freely after surgery.

Intra-articular injection of ADSCs

Thirty adult male New Zealand white rabbits were randomly divided into three groups: Normal (n = 6), OA (n = 12) and ADSC (n = 12). The OA and ADSC group underwent surgery and the Normal group untreated as controls in parallel. The rabbits were maintained under standard light/dark conditions and allowed free access to water and a standard commercial diet. ADSCs (passage 3) were prepared as cell suspension of 6×10^6 cells/200 μL in PBS. In the ADSC group, 200 μL cell suspension was administered to both hind legs by intra-articular injection using a medial parapatellar approach two weeks after surgery. The other groups received an equal volume of PBS in parallel. Half of the rabbits in each group were sacrificed six weeks post-surgery, and the remaining rabbits were sacrificed four weeks later.

Macroscopic examination and frozen tissue section preparation

The femoral condyles were collected from the sacrificed rabbits. The cartilage surfaces were macroscopically examined and photographed with a digital camera at six and ten weeks after surgery. The femoral condyles with articular cartilage were fixed with 4% paraformaldehyde for 48 h, decalcified with 12.5% EDTA for 4 weeks and dehydrated with 30% sucrose solution for 72 h. The articular cartilage was then isolated, frozen and embedded in OCT compound (Tissue-Tek, Tokyo, Japan). Finally, sections (7 μm thick) were cut in the sagittal plane with a freezing microtome (Leica, Heidelberger, Germany).

Histological and OA score analyses

The frozen sections were fixed again with acetone solution for 10 min, and then stained with hematoxylin and eosin (HE), Safranin O and fast green solution (Solarbio). The Osteoarthritis Research Society International (OARSI) scoring system was used to determine the extent of cartilage deterioration and the severity of knee OA, with scores ranging from 0 (normal) to 6 (>80% loss of cartilage). The modified Mankin criteria were used to assess cartilage damage, with scores ranging from 0 (normal) to 24 (the most severe osteoarthritis) [25, 26]. The evaluations were performed independently by two blinded observers and the scores were generated through evaluation of multiple serial sections from rabbit knees in the OA and ADSC groups.

Immunofluorescent analysis of tissue sections

The cartilage tissue sections from the OA and ADSC groups were incubated overnight at 4°C with anti-MMP13 primary detection antibody (Boster, Beijing, China, 1:200). The samples were then incubated at room temperature for 2 h with an anti-rabbit secondary detection antibody conjugated with CY3 fluorescent dyes (Boster, Beijing, China, 1:200) and viewed under a fluorescence microscope. The percentage of positive cells and mean optical density of MMP13 staining were analyzed using Image J software.

Statistical analysis

Data were presented as the mean ± SEM. All experiments were performed on at least three independent occasions. Statistical differences between groups were evaluated by Student's *t*-test or Mann-Whitney *U*-test. SPSS18.0 software was used for statistical analysis. $P < 0.05$ was considered to indicate statistical significance.

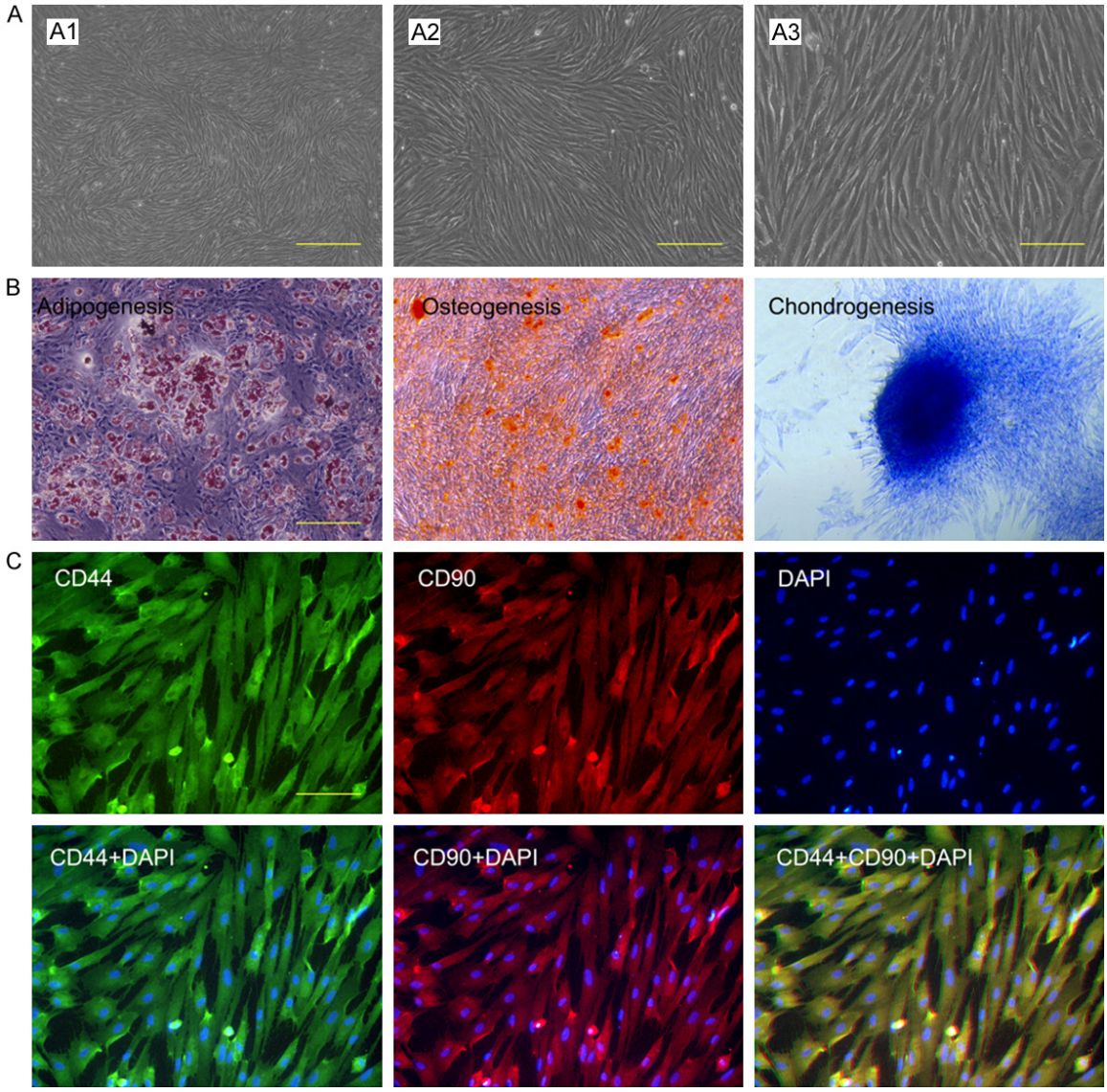

Figure 1. Morphological properties and identification of rabbit ADSCs. (A) Light microscopy images showing the characteristic fibroblast-like morphology of passage 3 ADSCs at 50×, 100×, and 200× magnification. Scale bars in (A1-A3) represent 400, 200 and 100 μm respectively. (B) The multipotency of ADSCs demonstrated by adipocytic differentiation (oil red O staining), osteogenic differentiation (alizarin red staining), and chondrogenic differentiation (toluidine blue staining). Scale bars represent 200 μm. (C) Photomicrographs of dual-immunofluorescence labeling of ADSCs for CD44 and CD90. Scale bars represent 100 μm.

Results

Culture and characterization of ADSCs and articular chondrocytes

The ADSCs (passage 3) multiplied to form a confluent monolayer, presenting a typical vortex-shaped structure at low magnification (50×, 100×), and a typical spindle-shaped and fibroblast-like morphology with parallel alignment at high magnification (200×) (**Figure 1A**). Oil red O staining for adipogenesis, alizarin red staining for osteogenesis and toluidine blue staining for chondrogenesis revealed that ADSCs (passage 3) exhibited multilineage differentiation potential with MSC properties (**Figure 1B**). The dual-immunofluorescent staining showed that was >95% of the cells were positive for the characteristic ADSC markers CD44 and CD90 (**Figure 1C**). The primary articular chondrocytes of adult rabbits grew slowly and were positive for toluidine blue and Col2a1 staining (**Figure 2**).

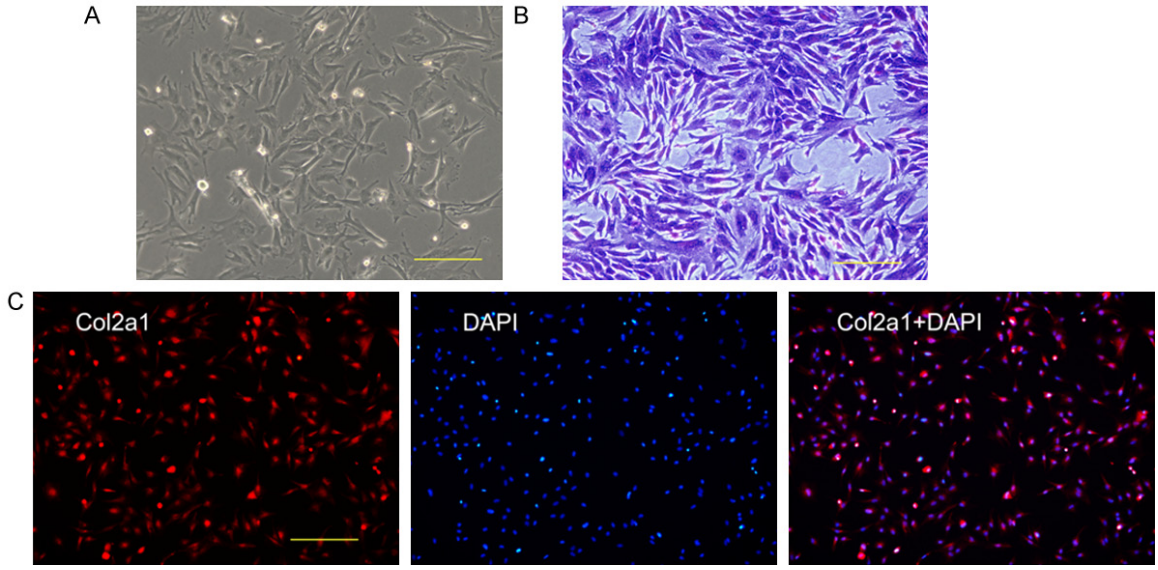

Figure 2. Morphological properties and identification of rabbit primary articular chondrocytes. A. Light microscopy images of rabbit primary articular chondrocytes. B. Images of toluidine blue staining. C. Images of Col2a1 labeling. Scale bars represent 200 μm.

ADSCs promote proliferation and migration of cocultured articular chondrocytes

The effect of ADSCs on articular chondrocyte proliferation was assessed in EdU incorporation assays. A marked increase in the number of EdU-positive cells was observed in the cocultured group (**Figure 3A**) and the percentage of EdU-positive cells was significantly higher than that in the control group (**Figure 3B**). These results suggested that ADSCs promote articular chondrocyte proliferation. In the migration experiment, the number of migratory chondrocytes cocultured with ADSCs was remarkably increased compared with that in the control group, indicating that ADSCs greatly accelerate chondrocyte migration (**Figure 3C**).

ADSCs upregulate the expression of molecular markers of chondrocyte redifferentiation in cocultured articular chondrocytes

In vitro culture of chondrocytes results in the dedifferentiation, which is characterized by the development of fibroblast phenotypes and reduced ability to produce cartilage ECM and increase the expression of molecular markers Col1a1, Col10a1 and Runx2 [27-29]. Redifferentiation of chondrocytes is indicated by increased expression of the molecular markers Col2a1, Aggrecan and Sox9 [28-31]. Col2a1 and Aggrecan are the main components in the ECM, and Sox9 is a crucial transcription factor for chondrogenesis and chondrogenic differentiation in cartilage development [32, 33]. Compared with the controls, increased mRNA levels of Aggrecan, Col2a1 and especially Sox9, were detected in the articular chondrocytes cocultured with ADSCs (**Figure 4A-C**). At the protein level, the expression of Aggrecan, Col2a1 and Sox9 was enhanced compared with that in the controls (**Figure 4D**, **4E**). In particular, Sox9 protein expression was increased by approximately 5-fold, which was consistent with the observed changes in mRNA expression. In addition, the expression of Col1a1, Col10a1 and Runx2 was decreased at the mRNA and protein levels in the cocultured group compared with that in the control group (**Figure 4F-J**).

Intra-articular injection of ADSCs ameliorated articular cartilage damage and retarded OA progression at different stages

The knee OA rabbit model was established using the ACLT and MMx method. Two weeks after surgery, rabbits received intra-articular injection of ADSCs. During the experiment, the rabbits injected allogeneic ADSCs did not display aberrant in body weight, movement, eating or other behaviors. The knee joints of the rabbits did not show swelling, redness or other adverse reactions. Macroscopic images were shown in **Figure 5A** and **5D**. In the normal

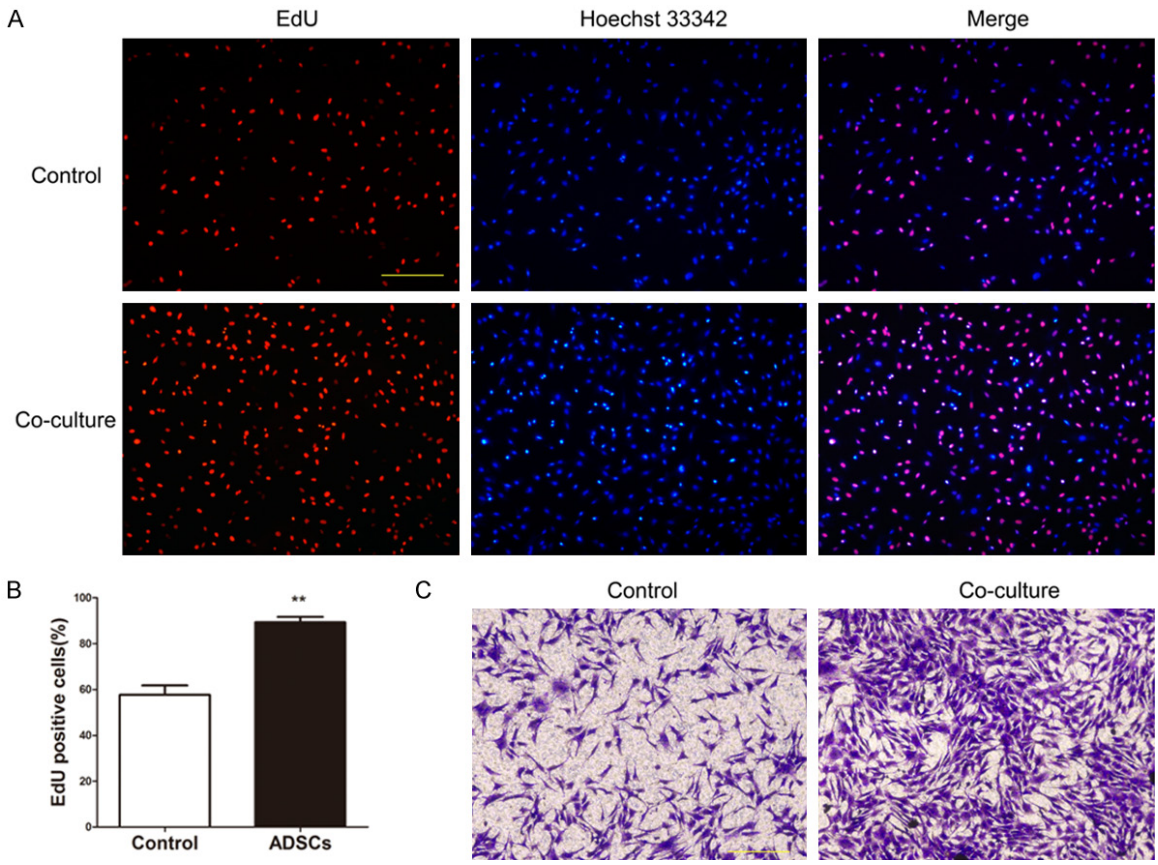

Figure 3. Effects of ADSCs on proliferation and migration of cocultured chondrocytes. A. EdU incorporation showing that ADSCs enhance the proliferation of cocultured chondrocytes. B. Quantification of proliferation of cocultured chondrocytes. Data represent the mean ± SEM of three independent experiments. C. Chondrocyte migration assays showing migrated chondrocytes stained by crystal violet. Scale bars represent 200 μm. $^{**}P < 0.01$.

group, the articular cartilage was intact and flat. At six weeks post-surgery, the articular cartilage of the medial femoral condyles in the model group was abraded and there were small defects in the surface. In the treatment group, the defects in the surface of the cartilage were almost completely reversed. At ten weeks post-surgery, the cartilage in the bilateral femoral condyles was evidently destroyed. The large lesions in the cartilage of OA rabbits were visibly repaired by ADSC treatment and the flat articular surface was partly recovered at this stage.

To further evaluate the severity of OA and the effects of ADSCs on cartilage damage, sections of articular cartilage were stained HE and Safranin O and fast green. At six weeks post-surgery, the large vertical fissures in the articular surface of the treatment group had disappeared compared with the OA group, whereas the surface of cartilage was not smooth compared with that in the normal group (**Figure 5A**). The staining of Safranin O and fast green was more intense compared with that in the normal group, suggesting that the proteoglycan content of the ECM was increased by ADSC treatment (**Figure 5A**). The OARSI and Mankin scores were significantly decreased in the treatment group compared with those in the OA group (**Figure 5B**, **5C**). At ten weeks post-surgery, histological staining demonstrated that most of the cartilage surface was worn and some subchondral bone was exposed in the model group (**Figure 5D**). Intra-articular injection of ADSCs greatly improved the cartilage defects, promoted cartilage regeneration and ECM synthesis, and reduced the OARSI and Mankin scores (**Figure 5E**, **5F**).

Intra-articular injection of ADSCs downregulated MMP13 in articular cartilage at different stages

The effects of ADSCs on the expression of the OA marker gene, MMP13, in articular cartilage

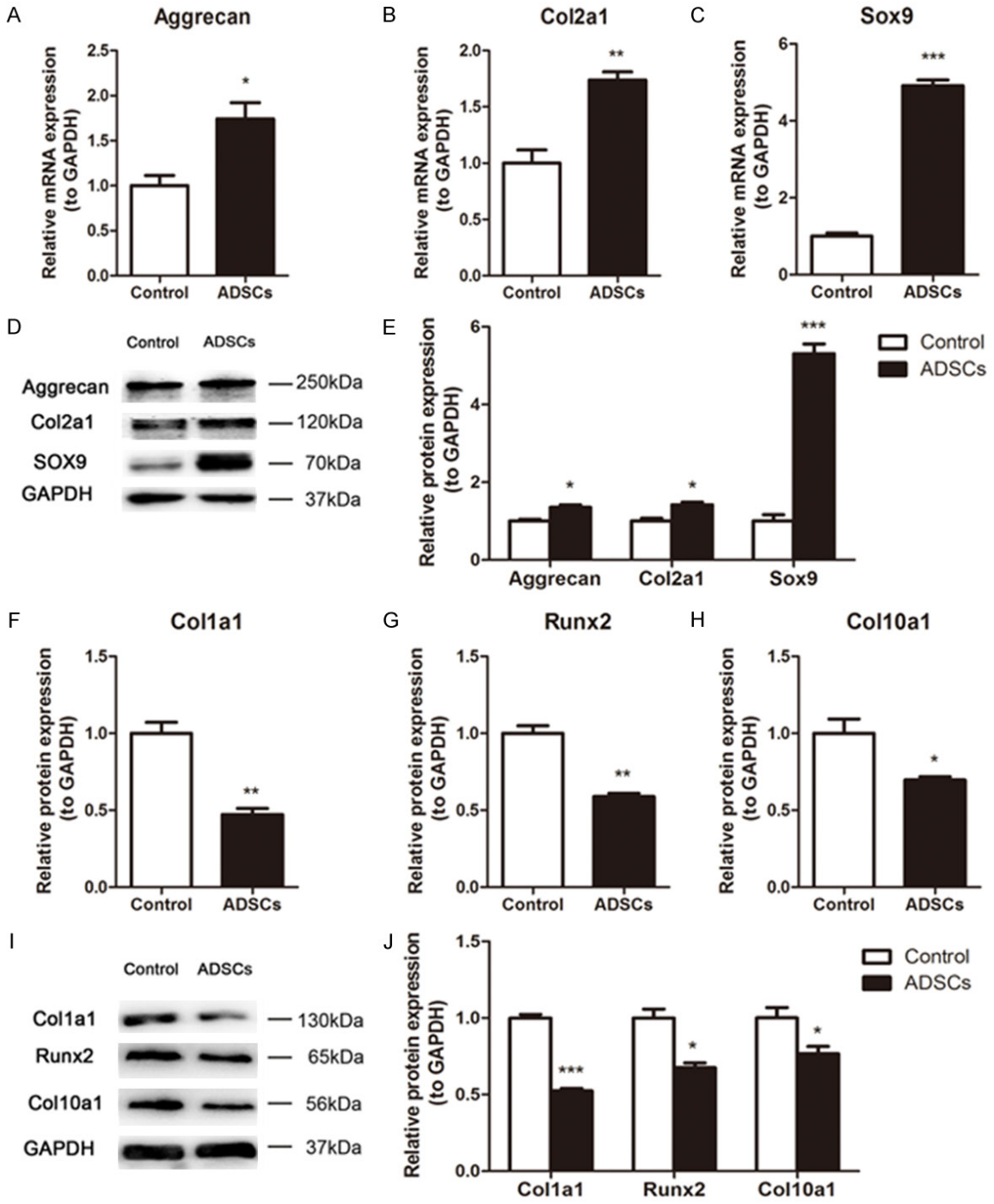

Figure 4. RT-qPCR and western blot analyses of molecular marker expression of chondrocyte redifferentiation for primary articular chondrocytes cocultured with ADSCs. A-C. Increased relative mRNA expression of Aggrecan, Col2a1 and Sox9 in chondrocytes cocultured with ADSCs. D, E. Increased relative protein expression of Aggrecan, Col2a1 and Sox9 in chondrocytes cocultured with ADSCs. F-H. Decreased relative mRNA expression of Col1a1, Col10a1 and Runx2 in chondrocytes cocultured with ADSCs. I, J. Decreased relative protein expression of Col1a1, Col10a1 and Runx2 in chondrocytes cocultured with ADSCs. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

were evaluated by immunofluorescent analyses. As shown in **Figure 6**, both the percentage of MMP13-positive articular chondrocytes and the mean optical density of MMP13 in the ADSC group were decreased compared with that in the OA group and significantly lower than

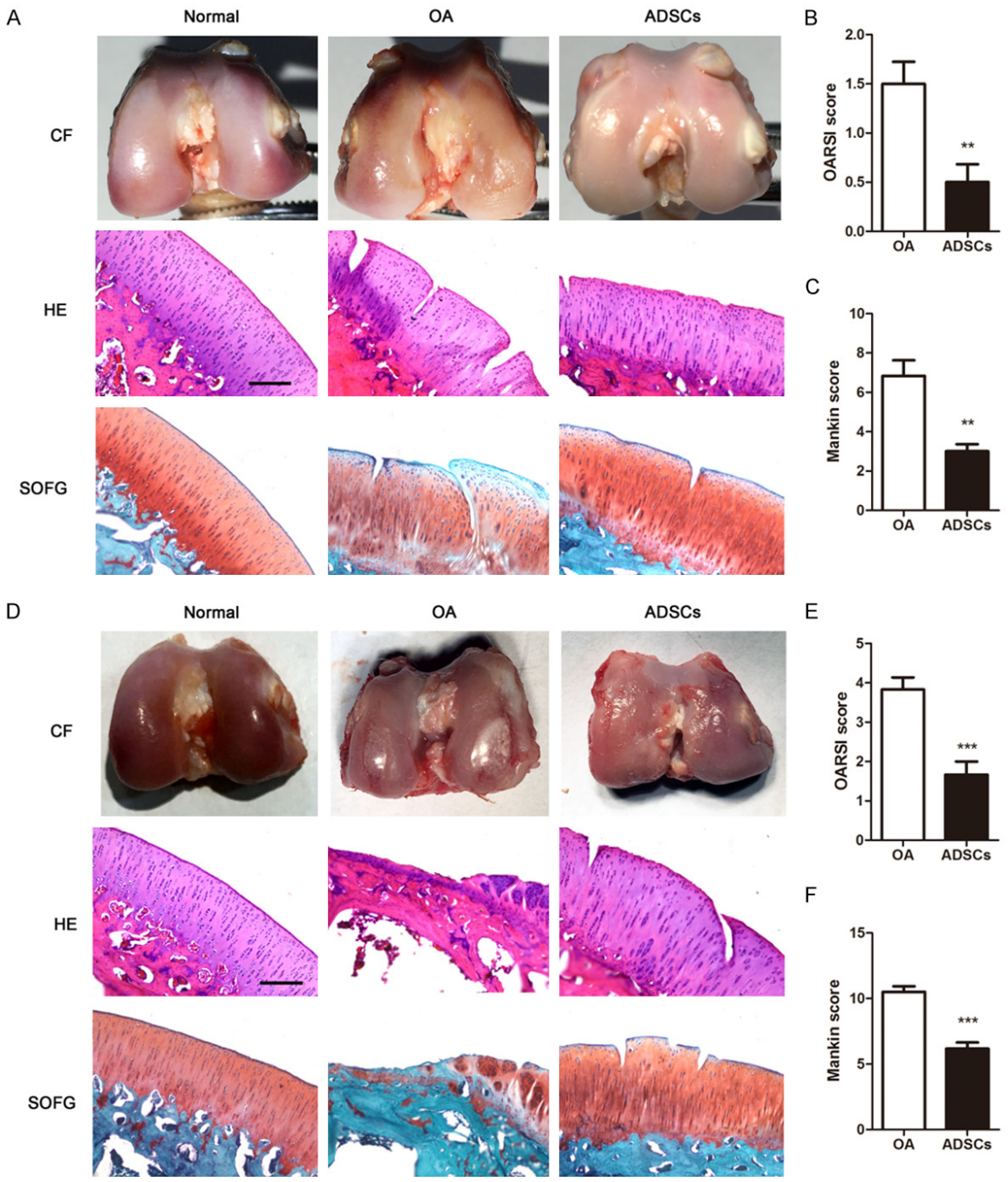

Figure 5. Macroscopic examination and histological assessment of intra-articular injection of ADSCs in OA rabbits at six and ten weeks post-surgery. A. Macroscopic examination of the FC and HE and SOFG staining six weeks post-surgery. B, C. OARSI and Mankin score assessment of the cartilage damage at six weeks post-surgery. D. Macroscopic examination and HE and SOFG staining of the FC at ten weeks post-surgery. E, F. OARSI and Mankin score assessment of the cartilage damage at 6 weeks post-surgery. FC: femoral condyle; HE: hematoxylin and eosin; SOFG: Saffron O and fast green. Scale bars represent 200 μm. $^{**}P < 0.01$, $^{***}P < 0.001$.

that in OA rabbits at six and ten weeks post-surgery. These results suggested that ADSCs greatly decreased the expression of MMP13 in articular cartilage *in vivo*.

Discussion

Recently, ADSCs have gained immense attention in the fields of regenerative medicine and

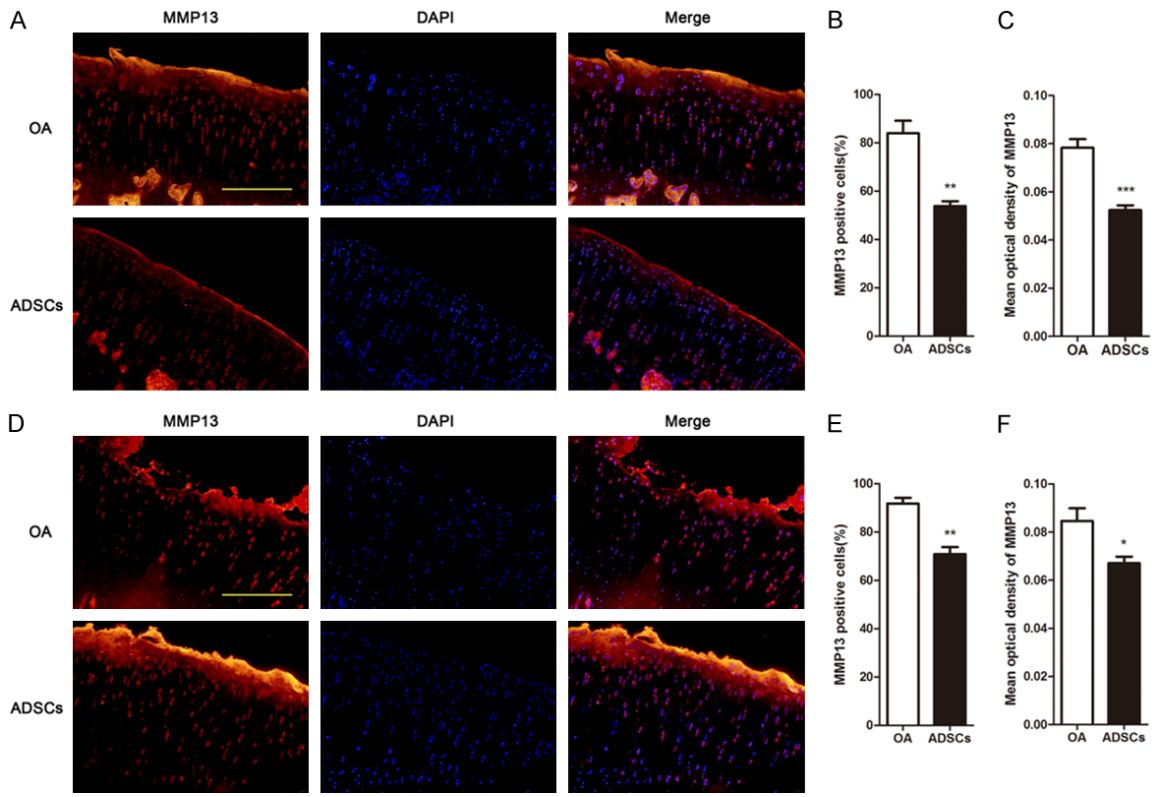

Figure 6. The expression of MMP13 in articular cartilage after intra-articular injection of ADSCs in OA rabbits established by surgery. (A) Immunofluorescence analysis of MMP13 expression in the articular cartilage of the medial femoral condyle six weeks post-surgery. (B, C) Quantification of MMP13-positive cells and the mean optical density of MMP13 based on the staining results of (A). (D) Immunofluorescence analysis of MMP13 expression in articular cartilage of the medial femoral condyle ten weeks post-surgery. (E, F) Quantification of MMP13-positive cells and the mean optical density of MMP13 based on the staining results of (D). Scale bars represent 200 μm. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

tissue engineering. In clinical practice, adipose tissue is easily obtained by lipectomy and liposuction procedures, which are less invasive and with a lower rate of donor morbidity compared with bone marrow aspiration [34, 35]. The yield of ADSCs from 1 mL lipoaspirate is 500-fold greater than the yield of BMSCs from 1 mL bone marrow aspirate [36]. Recently, ADSCs were prepared in the form of stromal vascular fraction (SVF) according to minimally manipulated restriction [37]. The SVF is a component of the lipoaspirate composed of different proportions of ADSCs, pericytes, vascular adventitia cells, fibroblasts, preadipocytes, monocytes, macrophages, and red blood cells [38]. While the SVF may produce favorable clinical outcomes in OA with minimal risk of side-effects, the effectiveness of ADSCs has been limited by the variability in the clinical data and the use of biological adjuvants [39]. Further *in vitro* and *in vivo* studies are required to fully elucidate the mechanisms underlying the effects of ADSCs for the treatment of OA.

In the current study, confluent cultured ADSCs (passage 3) were highly homogeneous in terms of morphological feature and immunological markers. The adipogenic, osteogenic and chondrogenic differentiation potential of the cells demonstrated the multilineage differentiation characteristic of stem cells. ADSCs are immunologically privileged because they do not express HLA-DR (major histocompatibility complex class II, MHC II) molecules and treatment with allogeneic ADSCs is safe and feasible [40, 41]. The chondrogenic differentiation potential and paracrine effect of MSC are critical for preclinical and clinical OA therapies based on engineered tissue transplantation and scaffold-free cell injection [42]. Nonetheless, the results of intra-articular injection of MSCs into osteoarthritic cartilage are controversial. Sato et al.

reported that some labeled-MSCs adhered to the surface and scattered within the cartilage, and Chiang et al. showed that a small number of the engrafted MSCs were evident in the articular surface cartilage, while some other studies showed that transplanted labeled-MSCs were located only in the synovial membrane and meniscus, but not in joint cartilage [14, 43-46]. Nevertheless, MSCs delivered into the articular cavity may play important roles in immunomodulation, anti-inflammation and chondro-protective effects in OA [47, 48].

In our study, we focused on the effects and changes in molecular expression patterns of ADSCs on articular chondrocytes and cartilage in OA treatment. Our *in vitro* results demonstrated that ADSCs significantly promoted the proliferation and migration of the primary articular chondrocytes when cocultured with these cells, suggesting that ADSCs may act on endogenous chondrocytes by secreting trophic factors. In articular cartilage, the decline in the capacity for cartilage repair is manifested as a decline in chondrocyte numbers leading to age-associated changes in ECM composition [49]. In our study, we found that cocultured ADSCs significantly upregulated the expression of articular chondrocyte redifferentiation markers and downregulated the expression of articular chondrocyte dedifferentiation markers at the mRNA and protein levels, suggesting that ADSCs was favorable in the formation of high-quality biomechanical articular cartilage *in vivo*.

In our *in vivo* experiment, the knee OA model was induced by surgery and the articular cartilage exhibited progressive damage. We randomly selected half of the OA model rabbits in the ADSC treatment group for intra-articular injection of ADSCs two weeks after surgery, which represents the initial or early stage of OA [23, 50]. Throughout the experiment, the allogeneic ADSCs did not cause any adverse events. In the previous studies used allogeneic ADSCs for OA treatment, some experiments did not specially mentioned that the ADSCs were allogeneic, or just described that the type of ADSCs was allogeneic and that no acute adverse events were observed [14, 15, 19, 51]. Some studies evaluated the safety of experimental animals by observing the phenomena of adverse reactions, such as swelling or joint redness [16-18]. In addition, intravenous delivery of allogeneic ADSCs for the treatment of OA has been reported [52]. The common conclusion from all the studies was that using allogeneic ADSCs was safe and did not cause adverse events. Therefore, intra-articular injeciton of allogeneic ADSCs is almost as safe as autologous ADSCs and has greater potential application for OA treatment in clinic. In the current study, we used allogeneic ADSCs to inject into the joint cavity of OA rabbits and found that it was safe and effective for the experimental animals.

Macroscopic observation and histological analyses at six weeks post-surgery showed that the OA group was at the early or middle stage of the disease; however, there were no apparent differences in the articular cartilage of the ADSC and normal groups. Furthermore, histological analysis revealed significant improvements in the condition of the articular cartilage in the ADSC group compared with that in the OA group. While the articular cartilage of OA rabbits at ten weeks post-surgery exhibited large lesions and the histological analyses indicated that the OA group was at late stage, ADSC treated rabbits exhibited slightly abrasion of the surface of articular cartilage, suggesting that intra-articular injection of ADSCs greatly retarded the progression of OA. The pathological destruction of OA cartilage is caused by an imbalance between the synthesis and degradation of the ECM [53]. Increased expression of MMP13, which is a major enzyme responsible for hydrolyzing collagen II, is a hallmark for OA [54]. The immunofluorescent analyses of MMP13 further verified that ADSCs repaired cartilage damage and altered OA-associated molecular expression. Our *in vivo* results at two time points demonstrated that ADSCs effectively alleviated rather than prevented OA progression. In contrast to our results, the intra-articular injection of expanded ADSCs for the treatment of clinical knee OA treatment exhibited a significant dose-dependent inhibition of disease progression; however, data from long-term patient follow-up over several years is scarce [13, 38, 55]. Our study indicates that repeat injection of ADSCs after a certain time may be more efficient for OA treatment.

Conclusions

In conclusion, rabbit ADSCs effectively promoted proliferation and migration of articular chondrocytes. The expression of molecular markers

of chondrocyte redifferentiation was increased by the secretion of ADSCs. Intra-articular injection of ADSCs evidently ameliorated articular cartilage damage and retarded OA progression at different stages in the rabbit OA model. This preclinical study demonstrates the effects and molecular mechanisms of allogeneic ADSCs on articular chondrocytes *in vitro* and articular cartilage *in vivo*, thus providing a favorable reference for clinical OA treatment of MSCs derived from adipose tissue.

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Disclosure of conflict of interest

None.

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