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Synovial mesenchymal stem cells effectively alleviate osteoarthritis through promoting the proliferation and differentiation of meniscus chondrocytes

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Abstract. – OBJECTIVE: To investigate the relationship between the meniscal defect area and OA progression and explore the effect and mechanism of SMSCs cell therapy in knee osteoarthritis (OA) rat model.

MATERIALS AND METHODS: For animal experiments, knee osteoarthritis (OA) model was constructed in Sprague Dawley (SD) rats by removing the medial meniscus of the right knee. Synovial mesenchymal stem cells (SMSCs) were engrafted by injecting into the right knee cavity. For *in vitro* experiments, CCK-8 assay was performed to evaluate the proliferation and differentiation of BMSCs and ATDC5 cells after co-cultured with SMSCs. qRT-PCR analysis was performed to detect the expressions of chondrogenic genes in BMSCs and ATDC5 cells after co-cultured with SMSCs. Western blot analysis was conducted to detect the phosphorylations of c-Jun N-terminal kinase (JNK) and extracellular regulated protein kinases (ERK) in MAPK signaling of BMSCs and ATDC5 cells. Enzyme-linked immunosorbent assay (ELISA) was performed to detect the serum levels of interleukin (IL)-1β, IL-1β, IL-6, IL-18 and C-reactive protein (CRP).

RESULTS: Results showed that meniscus damaged area is positively correlated to serum inflammatory factor levels. *In vitro* study showed that the proliferation and differentiation of BMSCs and ATDC5 cells were promoted after co-cultured with SMSCs. By co-culturing with SMSCs, the MAPK signaling pathway was activated and the expression of chondrogenic markers such as aggrecan (acan), SRY-related high mobility group-box gene 9 (sox9) and Type II collagen a1 (col2a1), was up-regulated both in BMSCs and ATDC5 cells. *In vivo* study showed SMSCs cell therapy significantly decreased serum inflammatory factor levels and protected cartilage by upregulating the expression of chondrogenic genes of meniscus chondrocytes derived from OA rats.

CONCLUSIONS: For the first time, we found the positive correlation between meniscal defect area and OA progression and demonstrated the effect and mechanism of SMSCs cell therapy in knee osteoarthritis (OA) treatment.

Key Words:
Synovial mesenchymal stem cells, Osteoarthritis, Meniscus chondrocytes.

Introduction

Osteoarthritis (OA) is a common chronic disease with an incidence of 6-40% in the general population[1,2]. With the aging of the population and the expansion of the obesity epidemic, it is expected that the incidence of OA will become the fourth leading cause of disability by the year 2020[3,4]. The pathogenesis of OA is complex and involves multiple tissues and processes, which are characterized by degeneration of articular cartilage and ligaments followed by a thickening of the subchondral bone and generation of osteophytes[5]. Knee OA is a relatively common type of OA, which is a bad consequence initiated by meniscus degeneration and damage mainly caused by sports, recreational activities and aging. Meniscus lesions often lead to potential disabling symptoms including swelling, pain from exercise and limited function, which are the principle risk factors for the progression of OA[6,7]. The major treatments of OA include exercises, analgesics or non-steroidal anti-inflammatory drugs[8-11]. Besides, many non-invasive treatments are used such as corticosteroid injections and hyaluronic acid (HA) injections before the last resort such as osteotomy or total knee arthroplasty[12-14]. In recent decades, stem cells have gradually become

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a potential way of human OA treatment[15,16]. Mesenchymal stem cells (MSCs), as a type of pluripotent cells that have the ability to differentiate into various cell lineages, have attracted much attention because of their easy access, safety and strong potential to differentiate into cartilage tissue[17,18]. In addition to their potential of differentiation, MSCs are also considered to have paracrine and immunomodulatory ability through releasing growth factors and cytokines[19,20]. The pathophysiology of OA is considered to be both degenerative and inflammatory. In that case, stimulating local growth, reducing immune response and tissue regeneration, may be beneficial and therapeutic[21]. Several studies have also investigated the transplantation of MSCs into the articular cartilage for the treatment of osteoarthritis.

The synovial membrane is a thin layer of connective tissue that connects the articular cavity, tendon sheaths, and bursae[22]. Since synovial fluid produced by synovium is important for lubrication, shock absorption, nutritional supplementation and waste transportation in joints, it is considered to play an important role in the dynamic balance of joints[22]. In addition, the synovial membrane is considered to include multipotent cells that contribute to tissue regeneration after injury[23]. It is reported that cells migrate from the synovial membrane after meniscal injury to regenerate the damaged tissue[24,25]. However, as the progenitor cells located in synovial joint, few studies have focused on whether SMSCs can alleviate OA symptoms by protecting OA cartilage damage and facilitating cartilage repair[26,27].

In our study, we investigated the relationship between meniscal defect area and OA progression using OA rats' model. Results indicate that meniscus damaged area is positively correlated to serum inflammatory factor levels. Further, we detected the proliferation and differentiation of BMSCs and ATDC5 cells after co-cultured with SMSCs. CCK-8 assay and qRT-PCR analysis were performed and found that co-culturing with SMSCs significantly increased the proliferation and chondrogenic differentiation. Results of Western blot analysis suggested that the MAPK signaling pathway of BMSC and ATDC5 cells was activated after co-cultured with SMSCs. Finally, we used SMSCs cell therapy to treat OA rats by direct infusion into the knee joint cavity. Corresponding analyses found that SMSCs cell therapy significantly decreased serum inflammatory factor levels and protected cartilage from OA damage. Our study not only demonstrated the potential of SMSCs cell therapy in the treatment of knee OA, but also suggested the role of MAPK signaling in SMSCs cell therapy in alleviating knee OA.

Materials and Methods

Cell Culture

Sprague Dawley (SD) rats were obtained from Animal Breeding Institute of Chinese Academy of Medical Sciences (Beijing, China). SMSCs were derived from Synovial tissues of knee joint of SD rats, while BMSCs were derived from bone marrow of SD rats. The SMSCs were acquired from the synovial tissues of rat using the enzymic methods. ATDC5 cells chondrogenic cell line were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Rockville, MD, USA) supplied with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Gibco) and incubated at 37° C in an atmosphere of 5% CO_2. Cells were passaged at 1:5 dilution when they reached 30-60% confluency using trypsin. Cells of generation 2-4 were used for further studies.

Animal Experiments

To investigate the effect of meniscus removal size on the progression of OA, 24 rats were randomly divided into three groups according to the removal area of the medial meniscus: 25% of medial meniscus, 50% of medial meniscus and the entire medial meniscus, 8 mice for each group. The surgery was performed under 0.5% chloral hydrate anesthesia and iodine sterilization. Under microscope, a lateral parapatellar skin incision was made longitudinally with ophthalmic scissors in the right knee joint at a level of 1 cm proximal to the patella. The patella was turned outward and fixed to expose joint capsule. The medial meniscus and tibial ligament were disconnected, followed by removing partial medial meniscus. The patella was then placed in the original position and sealed with a scalpel. After surgery, ampicillin (1.5 mg/kg) was injected intramuscularly for 3 days to prevent infection. To evaluate the efficiency of SMSCs cell therapy, 24 rats were randomly divided into two groups: control groups, OA model groups and treatment groups, 8 mice for each group. After removing the entire medial meniscus as described above,

rats were allowed to feed freely. 3 days after surgery, the treatment was performed by injecting 5×10^6 SMSCs.

Cytokine Measurement

At 4 and 8 weeks after surgery or SMSCs cell therapy, blood of rats was collected and centrifuged to separate serum in the supernatant. Enzyme-linked immunosorbent assay (ELISA) kits (Sigma-Aldrich, St. Louis, MO, USA) were used to assess the levels of IL-1β, IL-6, IL-18 and CRP.

RNA Extraction and qRT-PCR

After the culture plates were taken out, the cells were washed with PBS. After treatment, the total RNA of cells was extracted by using TRIzol reagent (Life Technologies, Waltham, MA, USA) according to the manufacturer's instructions. Samples were stored at room temperature for 30 min. The reverse transcription of cDNA was performed with a PrimeScript™ RT reagent Kit (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions. And for qRT-PCR, PCR primers were synthesized by GenePharma (Shanghai Gene Pharma, Shanghai, China) and sequences were listed in Table I. SYBR Premix Ex Taq II (TaKaRa, Otsu, Shiga, Japan) was used to detect the expression.

Western Blot Assay

Cells were lysed in a lysis buffer containing 10 mM Tris, pH 7.2, 150 mMNaCl, 5 mM EDTA, 0.1% SDS, 1% Triton X-100, and 1% deoxycholic acid. For Western blots, 20 mg of protein samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer onto polyvinylidene difluoride (PVDF) membranes. After blocking in 5% skim milk, membranes were incubated with rabbit antibodies against p-ERK, ERK, p-JNK, JNK and GAPDH (1:1000, Bioss, Beijing, China) overnight at 4°C followed by 1-hour incubation with secondary antibody (1:2000). Blots against GAPDH served as loading control.

CCK8 Assay

The CCK-8 kit (Dojindo, Kumamoto, Japan) was used to measure the cells proliferation according to the manufacturers' instructions. In brief, 5×10^3 cells were seeded in 96-well plates uniformly. After cells were treated with regulated medium, the medium was removed, and cells were washed with PBS solution for 3 times. Then, CCK8 dilution was added to the 96-well plates and incubated at 37°C in an atmosphere of 5% CO_2 for 2 hours. After incubation, the plates were taken out, and the cell proliferation was measured using multi-detection microplate reader. Further, the absorbance (OD) value at 490 nm of each well was detected.

Statistical Analysis

Unless otherwise indicated, all data were processed by SPSS 16.0 statistical software. (SPSS Inc., Chicago, IL, USA). All data were presented as mean ± SD. For comparisons of different groups, a one-way analysis of variance (ANOVA) and *t*-test were used, in which $*p < 0.05$, $**p < 0.01$ were considered statistically significant.

Results

Meniscus Defect Area is Positively Correlated with Serum Inflammatory Factor Levels in OA Rats

We first investigated the effect of meniscus removal size on the progression of OA. We removed different areas of the medial meniscus of the right knee, 25% of medial meniscus, 50% of medial meniscus and the entire medial meniscus, respectively. The detection of serum inflammatory factors in OA rats showed that the levels of inflammatory factors such as IL-1β, IL-6, IL-18 and C-reactive protein (CRP) significantly increased in different degrees after meniscus resection, and inflammatory factor levels were highest when the meniscus was completely removed (Figure 1A). Besides, the levels of IL-1β, IL-6, IL-18 and CRP were positively correlated with meniscus removal area. These results showed that meniscus defect area was positively correlated with serum inflammatory factor levels in OA rats, suggesting that meniscus defect size may play an important role in the OA progression.

Co-Culturing with SMSCs Significantly Enhanced the Proliferation and Differentiation of Chondrocytes

As discussed above, the retention area of meniscus plays an important role in the progression of OA; keeping the remaining area and reducing the degeneration rate of the meniscus may be potential therapies to alleviate OA. Here, we extracted primary SMSCs from SD rats and co-cultured with BMSCs and ATDC5 cells. CCK8-assay was performed on BMSCs and ATDC5 cells

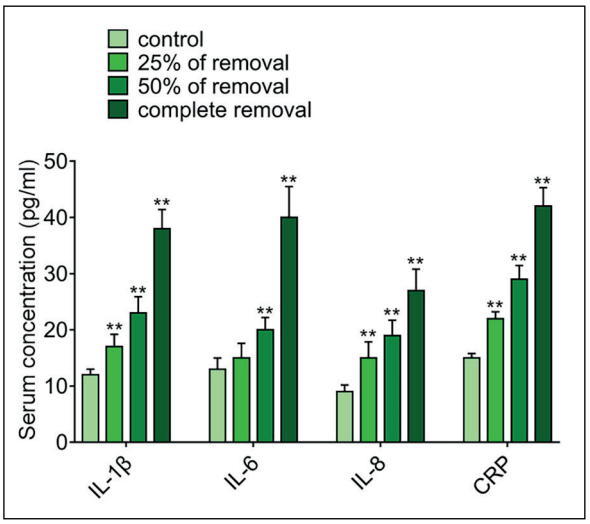

Figure 1. Meniscus defect area is positively correlated with serum inflammatory factor levels in OA rats. The serum levels of inflammatory factors in OA rats after removal of medial meniscus of different areas. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as *($p < 0.05$) or **($p < 0.01$).

after co-cultured with SMSCs for 3 days, and the results showed that co-cultured with SMSCs significantly increased the proliferation of BMSCs and ATDC5 cells (Figure 2A). We then performed qRT-PCR analysis to detect chondrogenesis-related genes including acan, sox9 and col2a1 after co-culturing for 3 days. The results showed that expressions of chondrogenic genes in BMSCs and ATDC5 cells were remarkably upregulated after co-cultured with SMSCs (Figure 2B, 2C).

SMSCs Elevated Cell Proliferation and Differentiation of Chondrocytes Through MAPK Signaling

MAPK signaling is a putative signaling pathway involved in cell proliferation and differentiation. To further evaluate the mechanism of SMSCs' effect on chondrocytes, western blot analysis was then performed to analyze the key proteins in the MAPK signaling. The results showed that phosphorylations of JNK and ERK in BMSCs and ATDC5 cells were significantly increased after co-cultured with SMSCs, indicating that activation of JNK/ERK pathway was involved in the chondrogenic differentiation of BMSCs and ATDC5 co-cultured with SMSCs (Figure 3A, 3B).

Blocking of MAPK by SCH772984 and SP600125 Suppresses Cell Proliferation and Differentiation of Chondrocytes Co-Cultured with SMSCs

In order to further clarify the role of MAPK signaling in cell proliferation and differentiation, SCH772984 (ERK1/2 inhibitor) and SP600125 (JNK inhibitor) were used to inhibit MAPK. SMSCs were co-cultured with BMSCs and ATDC5 cells, respectively. And all groups were treated with SCH772984 and SP600125 at the concentration of 40 μg/l. CCK8 assay results revealed that cell proliferation was decreased significantly after treated with SCH772984 and SP600125 for 3 days (Figure 4A, 4B). Then, qRT-PCR was carried out to investigate chondrogenic differentiation of BMSCs and ATDC5 co-cultured with SMSCs. The results showed that the expression levels of sox9 and col2a1 were significantly decreased in BMSCs treated with SCH772984 and SP600125 (Figure 4C, 4D). Similarly, in ATDC5 cells, the expressions of chondrogenic genes were remarkably suppressed (Figure 4E, 4F). Taken together, MAPK pathway plays an essential role in the chondrogenic differentiation of BMSCs and ATDC5 co-cultured with SMSCs.

SMSCs Cell Therapy Decreased Inflammatory Factor Levels and Protected Cartilage from OA Damage

After that we found co-culturing with SMSCs significantly promoted chondrogenesis *in vitro*, we speculated whether SMSCs cell therapy could play a role in OA rats' model. OA rats were treated with SMSCs cell therapy by direct infusion into the knee joint cavity followed by a detection of serum inflammatory factor levels (Figure 5A). Intriguingly, after 4 weeks of treatment, IL-1β, IL-6, IL-18 and CRP levels were significantly lower than OA control groups ($p < 0.05$). At 8 weeks, the levels of inflammatory factors further increased in the OA control groups, but OA treated groups displayed a significantly decreased expression of those inflammatory factors. Later, we extracted RNA from meniscus chondrocytes of OA rats and performed qRT-PCR analysis to detect the expression of chondrogenic genes. Results suggested that the expressions of acan, sox9 and col2a1 were significantly upregulated after SMSCs cell therapy for 8 weeks (Figure 5B). These evidences suggested that SMSCs cell therapy

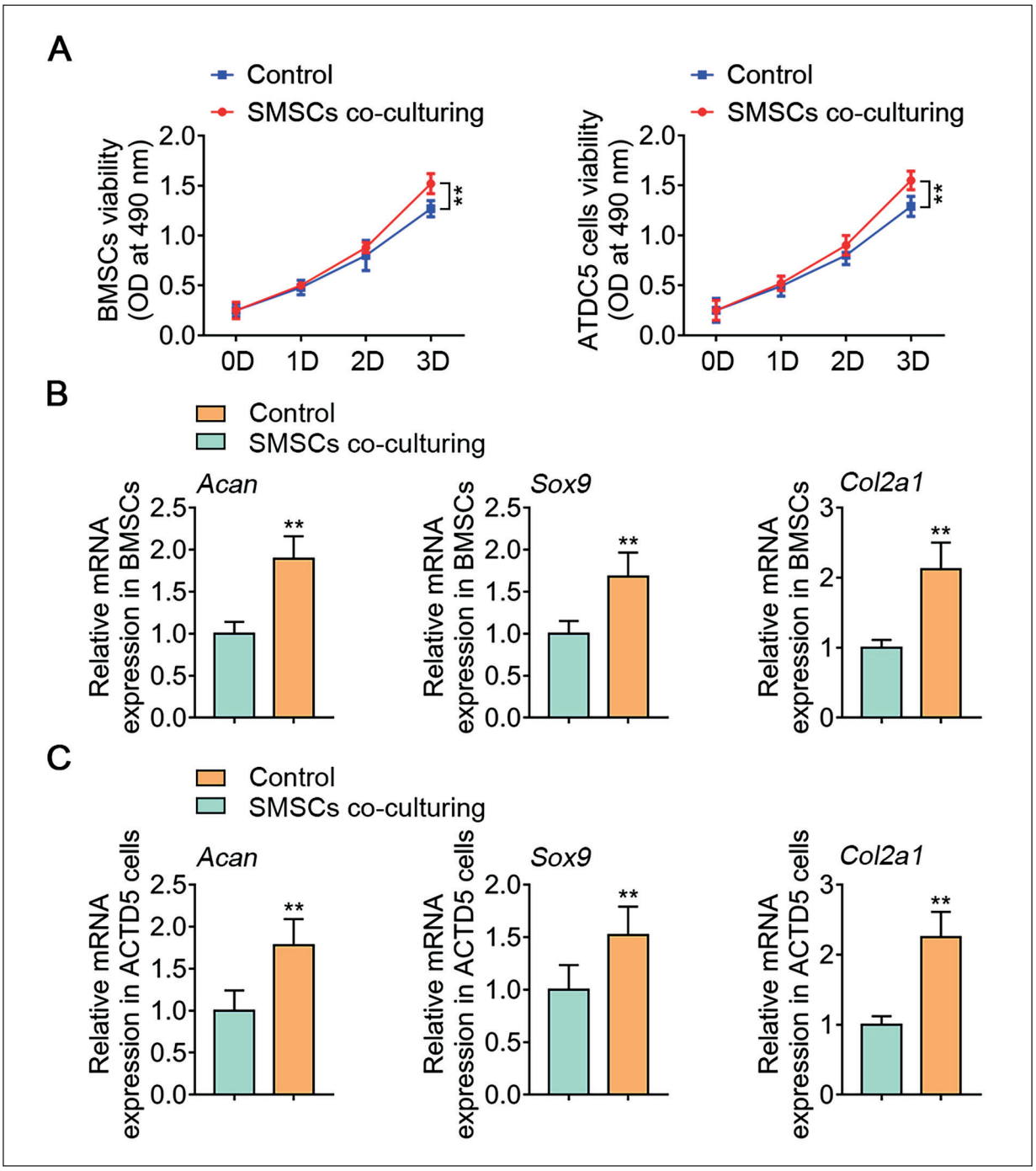

Figure 2. Co-culturing with SMSCs significantly enhanced the proliferation and differentiation of chondrocytes. **(A)** Absorption at 490 nm of BMSCs and ATDC5 cells co-cultured with SMSCs detected by CCK-8 assay at 1 d, 2 d and 3 d. Relative mRNA expression levels of Acan, Sox9 and Col2a1 in **(B)** BMSCs and **(C)** ATDC5 cells co-cultured with SMSCs detected by qRT-PCR analysis. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as *($p < 0.05$) or **($p < 0.01$).

alleviated inflammation resulted from OA and protected cartilage from OA damage, which may be a potential therapy for a better outcome of OA treatment.

Discussion

There exists a complicated connection between meniscus injury and OA pathogenesis. Gener-

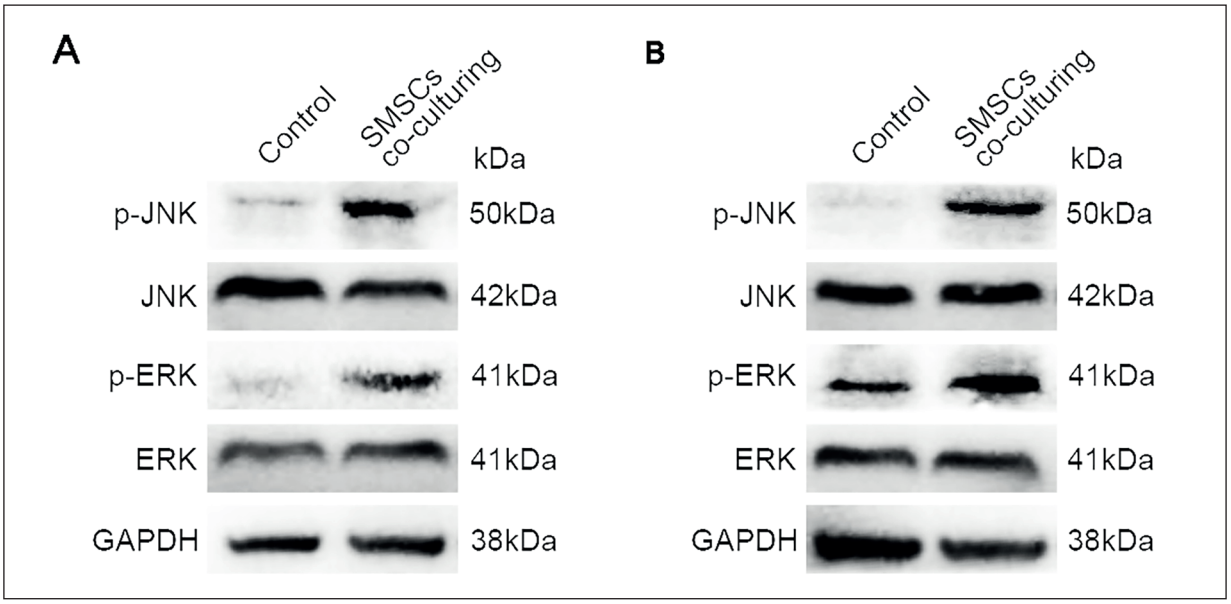

Figure 3. Blocking of MAPK by SCH772984 and SP600125 suppresses cell proliferation and differentiation of chondrocytes co-cultured with SMSCs. **A-B,** Absorption at 490 nm of BMSCs and ATDC5 cells co-cultured with SMSCs detected by CCK-8 assay after treated with SCH772984 and SP600125 for 3 days.

al view was that meniscal defect is a potential risk factor for the development and progression of OA. Once the meniscus lost its key function in the knee joint, an increase in biomechanical load on the articular cartilage may result in loss of cartilage and bone alterations including trabecular bone changes, increased bone mineral density, showing that a vicious circle of knee osteoarthritis is taking place[28-32]. However, there was no direct evidence that the damage of the meniscus contribute to the occurrence of OA[33]. In our study, we found that meniscus defect is positively correlated with serum inflammatory factor levels in OA rats, which suggested that the larger meniscus defect area may promote OA-related inflammation. Besides, since the severity of meniscal defect is positively correlated with the OA progression, we have strong reasons to protect meniscus tissue as much as possible to provide substantial meniscal function and prevent OA progression. These findings further refined the relationship between meniscal defects and OA progression and laid the foundation for future study.

In recent years, MSCs have been widely used as important sources of seed stem cells in the reconstruction of various damaged tissues[34,35]. SMSCs are a class of MSCs and are considered to have better proliferation and chondrogenic differentiation abilities than BMSCs[36,37]. The role of MSCs in the treatment of osteoarthritis was well illustrated in recent studies[38]. Ding et al[39] have demonstrated that MSCs from meniscus have a strong ability in chondrogenic differentiation, whereas BMSCs showed a greater ability of osteogenic potential. According to the previous reports, SMSCs showed good capacity for self-renewal and proliferation, and can be extended to the 5th or 8th generation without significant de-differentiation[40]. In this study, we used passage 2 SMSCs with homogeneous appearance and high purity. SMSCs have already been reported to play an important role in regeneration and differentiation of meniscus *in vitro*[41-43]. However, a few studies have investigated the role of SMSCs cell therapy in the treatment of OA *in vivo*. Our findings demonstrated that SMSC can also protect the meniscal degeneration and OA progression *in vivo*, which filled the gaps in previous research fields.

A previous study has demonstrated a engraftment-inducing tissue repair by using precartilaginous stem cells (PSCs) in OA rats; McKinney et al[44] found PSCs alleviated OA by inhibiting inflammation-related Notch 1 signaling pathways. Another study investigated the efficacy of intra-articular injection of MSCs in the treatment of early OA[44]. In this study, we showed that

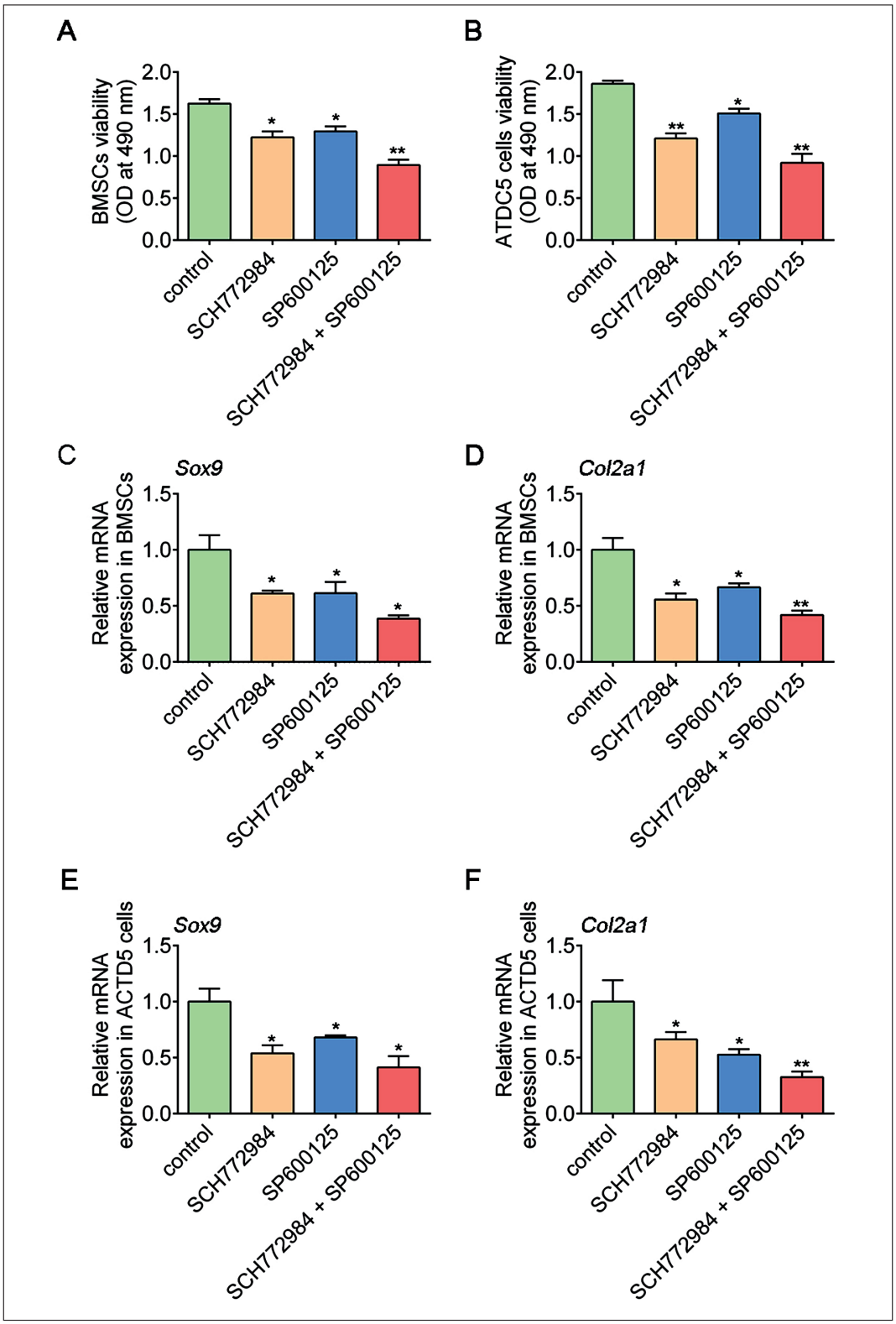

Figure 4. SMSCs elevated cell proliferation and differentiation of chondrocytes through MAPK signaling. Western blots of the phosphorylation of JNK and ERK in **(A)** BMSCs and **(B)** ATDC5 cells co-cultured with SMSCs. Relative mRNA expression levels of Sox9 **(C)** and Col2a1 **(D)** in BMSCs detected by qRT-PCR analysis. Relative mRNA expression levels of Sox9 **(E)** and Col2a1 **(F)** in ATDC5 cells detected by qRT-PCR analysis. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as *($p < 0.05$) or ** ($p < 0.01$).

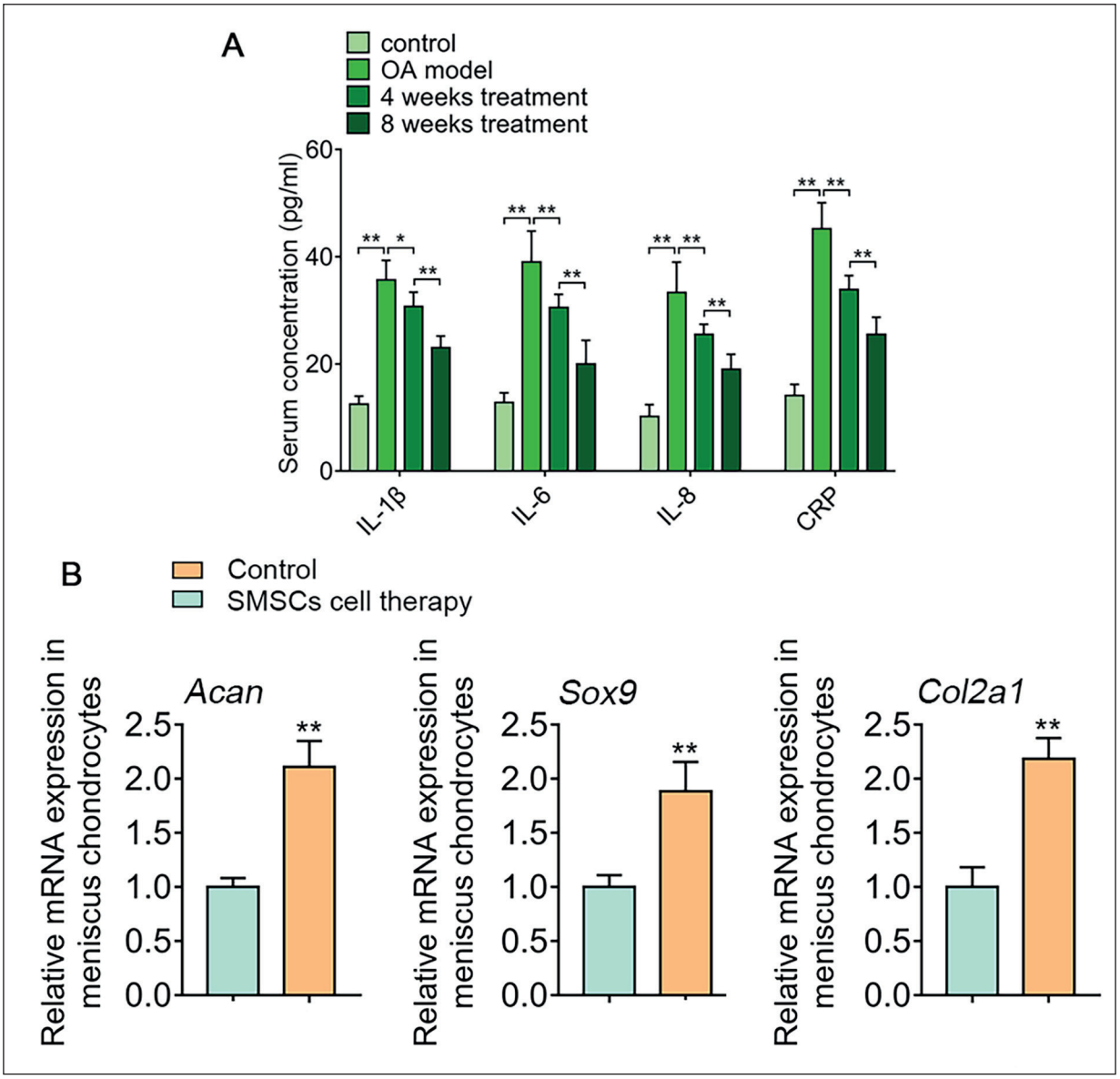

Figure 5. SMSCs cell therapy decreased inflammatory factor levels and protected cartilage from OA damage. **A,** The serum levels of inflammatory factors in OA rats after treated with SMSCs cell therapy for 4 weeks and 8 weeks. **B,** Relative mRNA expression levels of Acan, Sox9 and Col2a1 in meniscus chondrocytes in OA rats after treated with SMSCs cell therapy for 8 weeks. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as ($p < 0.05$) or **($p < 0.01$).

co-culturing with SMSCs significantly promoted the proliferation of BMSCs and ATDC5 and up-regulated the expression of chondrogenic genes *in vitro*. In the attempt to elucidate the mechanism of SMSCs co-culturing, we evaluated how SMSCs treatment altered MAPK signaling pathway, which is considered to be the key pathway regulating cell proliferation and differentiation. We showed that BMSCs and ATDC5 cells displayed pronouncedly higher phosphorylations of JNK and ERK levels after co-cultured with SMSCs, indicating the involvement of MAPK signaling in SMSCs-induced chondrogenic differentiation. *In vivo* study showed that SMSCs cell therapy of OA rats significantly reduced inflammatory factor levels and protected cartilage from OA damage, which further demonstrated the essential potential of SMSCs cell therapy in protecting meniscus cartilage damage and in alleviating the OA progression.

Conclusions

Taken together, we found the positive correlation between meniscal defect area and OA progression and demonstrated the effect and mechanism of SMSCs cell therapy in knee osteoarthritis (OA) treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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