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# Micro-fragmented adipose tissue regulated the biological functions of osteoarthritis synoviocytes by upregulating MiR-92a-3p expression

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ABSTRACT

Apart from the treatment potential of micro-fragmented adipose tissue (MF) in joint diseases, what's less clear is the mechanism of MF on Osteoarthritis (OA). Synoviocytes isolated from synovium tissues of 11 knee joint OA patients were identified and co-cultured with MF collected by Lipogems®. Cytokines and mRNA levels in synoviocytes were detected by enzyme-linked immunosorbent assay (ELISA) and quantitative reverse transcription polymerase chain reaction (qRT-PCR). Cell viability, apoptosis and apoptosis-related protein expression of Tumor Necrosis Factor-α (TNF-α)-activated synoviocytes were detected by cell counting kit-8, flow cytometry and western blot, respectively. The rescue experiments were conducted to verify the causal relationship of MF and miR-92a-3p. The relationship between miR-92a-3p and KLHL29 was verified by bioinformatics analysis, qRT-PCR, dual-luciferase reporter assay and western blot. OA synoviocytes were composed of synovial fibroblasts and synovial macrophages. After co-cultivation of synoviocytes and TNF-α, the levels of Interleukin (IL)-8 and hyaluronic acid (HA) appeared a few changes, and those of chemotactic cytokine ligand (CCL) 2, CCL3, CCL5 and matrix metalloproteinases (MMP)-9 were downregulated, while the levels of Tissue Inhibitor of Metalloproteinases (TIMP)-1, IL-10 and Prostaglandin E2 ($PGE_2$) were up-regulated. Co-culture of MF and activated synoviocytes reversed the above-mentioned effects regulated by TNF-α and reduced the mRNA levels of inflammatory factors. However, miR-92a-3p inhibitor overturned the reversal. KLHL29 was the target gene of miR-92a-3p and its expression was suppressed in activated synoviocytes co-cultured with MF, which was reversed by down-regulated miR-92a-3p. Collectively, MF regulated the biological functions of OA synoviocytes by upregulating miR-92a-3p expression.

## 1. Introduction

Osteoarthritis (OA) is a chronic arthritis disease with joint swelling, pain or dysfunction as the main clinical manifestations (Glyn-Jones et al., 2015). The pathological features of synovial inflammation include abnormal synovial proliferation, infiltration of macrophages and lymphocytes, and neovascularization, which acted as an important role in the pathogenesis of OA (Bondeson et al., 2010). The inner layer of the synovium is distributed with synovial macrophages (SM) and synovial fibroblasts (SF) (Scanzello and Goldring, 2012). The activation of SM secretes a variety of inflammatory factors, chemokines and growth factors which can activate and induce local SF to produce interleukins, prostaglandins and matrix metalloproteinases (MMPs) (Burrage et al., 2006; Wojdasiewicz et al., 2014). These inflammatory mediators diffuse to the joint fluid, cartilage matrix and cartilage cells, and aggravate the condition of OA through cartilage degradation (Kapoor et al., 2011).

The current treatment of OA is limited to relieve superficial symptoms and inflammation which cannot solve the problem fundamentally. In recent years, adipose-derived mesenchymal stem cells (AD-MSC) have made great progress in the treatment of rheumatic diseases by virtue of the large quantity, strong differentiation and repair capabilities (Minteer et al., 2013). AD-MSC exerts assorted effects such as immune regulation, anti-inflammation and chemotaxis by cytokine secretion and direct interaction with cells. However, the blood and oil in the obtained fat could cause infection and inflammation, and the acquisition of AD-MSCs was complicated (Mahmoudifar and Doran, 2015). To address this point, Lipogems® (LG), a device for collecting micro-fragmented adipose tissue (MF), was found to be useful in clinical practice (Tremolada et al., 2016). Studies have proven that MF obtained by LG contains functional AD-MSCs which can release bioactive cytokines to initiate the immune

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regulation against the target cells (Ceserani et al., 2016). Studies have also compared the effects of AD-MSC and MF in the treatment of OA, and found that MF is more suitable for clinical application than AD-MSC (Paolella et al., 2019; Tofino-Vian et al., 2018). However, current efforts to dissect the mechanism of MF in OA synovium were insufficient.

In recent years, the therapeutic potential and role of microRNAs (miRNAs) in OA have been revealed one after another (Coutinho de Almeida et al., 2019; Wu et al., 2019). The expression of miR-92a-3p is reduced in exosomes secreted by OA chondrocytes, and overexpression of miR-92a-3p can enhance cartilage formation and inhibit cartilage degradation (Mao et al., 2018). Not only that, Zhang et al. pointed out that miRNAs play pivotal regulatory roles in the cartilage formation of human adipose-derived stem cells (hADSCs), and uncovered that miR-92a expression is significantly up-regulated in hADSCs (Zhang et al., 2012). Therefore, we conjectured that MF may affect the biological behaviors of synoviocytes by regulating the expression of miR-92a-3p.

## 2. Materials and methods

### 2.1. Sample collection

Synovial tissues were obtained from 11 knee joint OA patients treated in Zhejiang Academy of Medical Sciences from January 2019 to December 2019. All patients met the classification criteria of the American College of Rheumatology for OA (Hochberg et al., 2012). The written informed consents were signed by all patients. The study was authorized by Ethics Committee of Zhejiang Academy of Medical Sciences with the following reference number: F201812015.

The collection of MF was conducted as previously described (Bianchi et al., 2013). Briefly, the collected adipose tissues were immediately put into the LG (Lipogems International SRL, Italy) processing device. The LG system processed the collected adipose tissues in a closed and sterile environment, and transformed the tissues into 1-2 millimeter (mm) micro-fragments. In addition, the LG device removed the inflammatory components and coarse fibers in the fat, thereby avoiding infection and inflammation and improving the viability of the fat.

### 2.2. Culture of synoviocytes

Synoviocytes were obtained from synovial tissues of knee joint OA patients and the isolation method was referred in a previous research (Sluzalska et al., 2017). The synovial tissue samples were minced and placed in phosphate buffered saline (PBS) (C0221A, Beyotime, China) with 0.1 % trypsin (P4201, Beyotime, China) for 30 min. Then the tissues were digested with 0.1 % collagenase (C0130, Merck, Germany) for 4−6 hours (h) and then filtered through a filter membrane. Finally, the obtained cells were cultured in Dulbecco's modified Eagle medium (DMEM) (11965092, Gibco, USA) containing 10 % fetal bovine serum (FBS) (10091, Gibco, USA) and 1% penicillin-streptomycin (15140-122, Gibco, USA) in an incubator at 37 °C with 5% $CO_2$ (Forma Steri-Cycle, Thermo Scientific, USA). The cultured OA synoviocytes were photographed by optical microscope (BX53 M, Olympus, Japan, 100×), which were spindle-shaped and star-shaped.

### 2.3. Co-culture experiments

To investigate the paracrine interaction between synoviocytes and MF, we established a co-culture system. Co-culture experiments were performed by the method described previously (Paolella et al., 2019). The co-culture system was separated from the upper and lower compartments by a 0.4 μm pore. OA synoviocytes were seeded in the lower compartment at a density of $1 \times 10^5$ cells/well and MF at a concentration of 0.1 g/mL was placed in Transwell insert (3412, corning, USA). Co-culture system was grown in DMEM supplemented with ascorbic acid (A5960, Merck, Germany), proline (P0380, Merck, Germany), and sodium pyruvate (S8636, Merck, Germany). Mono-cultured synoviocytes and MF in the same conditions were regarded as controls.

### 2.4. Groups

For detecting the effects of TNF-α (T6674, Merck, Germany) on biological functions of synoviocytes, the experiments were constructed in four groups: Syn. group (normally cultured synoviocytes), TNF-α-1 group (synoviocytes were treated with 1 ng/mL TNF-α for 24 h), TNF-α-5 group (synoviocytes were treated with 5 ng/mL TNF-α for 24 h) and TNF-α-10 group (synoviocytes were treated with 10 ng/mL TNF-α for 24 h).

To determine the effects of co-culture system on biological functions of synoviocytes stimulated by TNF-α, we used TNF-α (10 ng/mL) to amplify the inflammation and constructed the co-culture system. The experiments were established within three groups: Syn. group (normally cultured synoviocytes), Syn. + TNF-α group (synoviocytes were treated with 10 ng/mL TNF-α for 24 h) and Syn. + TNF-α + MF group (synoviocytes were treated with 1 ng/mL TNF-α for 24 h and then co-cultured with MF).

To examine the effects of miR-92a-3p on biological functions of synoviocytes, the experiments were set up in three groups: Syn. + TNF-α group (synoviocytes were treated with 10 ng/mL TNF-α for 24 h), Syn. + TNF-α + MF group (synoviocytes were treated with 1 ng/mL TNF-α for 24 h and then co-cultured with MF) and Syn. + TNF-α + MF + I (inhibitor) group (synoviocytes transfected with miR-92a-3p I were treated with 10 ng/mL TNF-α for 24 h and then co-cultured with MF).

To reveal the relationship among KLHL29, inflammation, and apoptosis in synoviocytes, the experiments were set up in three groups: TNF-α, TNF-α+MF, TNF-α+MF + KLHL29 (synoviocytes transfected with KLHL29 plasmid were treated with 10 ng/mL TNF-α for 24 h and then co-cultured with MF).

### 2.5. Quantification of secreted factors

Enzyme-linked immunosorbent assay (ELISA) kits including CXCL8/IL-8 (#KHC0081, Thermo Fisher, USA), CCL2/MCP-1 (#BMS281, Thermo Fisher, USA), CCL3/MIP-1α (#BMS2029INST, Thermo Fisher, USA), CCL5/RANTES (#EHRNTS, Thermo Fisher, USA), MMP-9 (#BMS2016-2, Thermo Fisher, USA), TIMP-1 (#EH456RB, Thermo Fisher, USA), Hyaluronic acid (HA) (ml064280, mlbio, China, http://www.mlbio.cn/), IL-10 (#BMS215-2, Thermo Fisher, USA) and Prostaglandin E2 ($PGE_2$, #KHL1701, Thermo Fisher, USA) were applied to quantify the concentration of cytokines in the supernatant. The procedure was conducted according to the manufacturer's instructions. Briefly, the capture antibody was coated on a 96-well plate at room temperature overnight. The next day, the supernatant was diluted with Reagent Diluent to an appropriate concentration and then added to each well. Detection antibody, streptavidin-HRP, and substrate solution were added to each well in turn. Finally, 50 μL stop solution was used to stop reaction. Multi-detection reader (SpectraMax5, Molecular Devices, USA) was applied for detecting absorbance at 540 nm.

### 2.6. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was collected from OA synoviocytes using TRIzol (15596026, Invitrogen, USA). Reverse transcription was conducted using reverse transcription kit (RR047A, Takara, Japan). The mRNA expressions of CXCL8/IL-8, CCL2/MCP-1, CCL3/MIP-1α, CCL5/RANTES, TWIST1, KLHL29, CACNA1C and miR-92a-3p were detected by qPCR Quantitation Kit (E021001, GenePharma, China) or miRNAs qPCR Quantitation Kit (E01007, GenePharma, China) and analyzed by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Parameters included the pre-denaturation (95℃ for 3 min), followed by 40 cycles of the denaturation (95℃ for 30 s) and the final extension (60℃ for 40 s). The

primers were listed in Table 1. β-actin or U6 was used as an internal control.

### 2.7. Cell viability assay

We detected the cell viability using cell counting kit-8 (CCK-8) assay kit (C0037, Beyotime, China). After cells were processed differently according to the experimental requirements, 10 μL CCK-8 solution was added to each well of the 96-well plate. The plate was placed in the incubator and cultured for 2 h. A multiple detection reader (SpectraMax5, Molecular Devices, USA) was applied to detect the absorbance at 450 nm.

### 2.8. Flow cytometry

Cell apoptosis was detected by flow cytometry using Annexin V-FITC apoptosis detection kit (C1062S, Beyotime, China). 195 μL Annexin V-FITC binding solution was bound with $3 \times 10^5$ cells that were mixed with 10 μL Annexin V-FITC and 5 μL Propidium iodide (PI). Following the incubation for 15 min in the dark, the sample was collected by Flow cytometer (DxFLEX, Beckman, USA) and the results were analyzed by CytExpert (Beckman, USA).

### 2.9. Western blot

Western blot was conducted according to a previously reported research (Alegria-Schaffer et al., 2009). Proteins were obtained using RIPA buffer (P0013B, Beyotime, China) supplemented with protease inhibitors (P1005, Beyotime, China) and Phenylmethylsulfonyl Fluoride (PMSF) (ST505, Beyotime, China). The cell lysates were quantified by the BCA kit (P0011, Beyotime, China) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), respectively. After the electrophoresis, the proteins were transferred into the polyvinylidene fluoride (PVDF) membrane (160-0184, Bio-Rad, USA). After blocking with 5% skim milk for 1 h, the membrane was incubated with primary antibodies against Bcl-2 (1:1000, 26 kDa, ab196495, Abcam, UK), Bax (1:1000, 21 kDa, ab53154, Abcam, UK), cleaved caspase-3 (1:1000, 17 kDa, ab49822, Abcam, UK), KLHL29 (1:1000, 94 kDa, ab201086, Abcam, UK) and β-actin (1:1000, 42 kDa, ab8226, Abcam, UK) overnight. After washing, the membrane was incubated with the secondary antibody, goat anti-rabbit (1:10000, ab205718, Abcam, UK) or goat anti-mouse (1:10000, ab6789, Abcam, UK). ECL luminescence kit (PE0010, Solarbio, China) and gel imaging system (FluorChem FC3, Alpha, USA) were applied to expose the membrane and visualize the protein band, respectively. ImageJ2x (Rawak Software, Germany) was applied to count results.

**Table 1**
Primers were used in this study.

| List of oligonucleotide sequences | 5′–> 3′ |
|---|---|
| **Primers for PCR** | |
| CXCL8/IL-8 Forward | AGGACAAGAGCCAGGAAGAA |
| CXCL8/IL-8 Reverse | GGGTGGAAAGGTTTGGAGTATG |
| CCL2/MCP-1 Forward | CTGTGCCTGCTGCTCATAG |
| CCL2/MCP-1 Reverse | CTTGCTGCTGGTGATTCTTCT |
| CCL3/MIP-1α Forward | CCAAGTCTTCTCAGCGCCAT |
| CCL3/MIP-1α Reverse | GAATCTTCCGGCTGTAGGAGAAG |
| CCL5/RANTES Forward | GTGCCCACGTCAAGGAGTAT |
| CCL5/RANTES Reverse | GGGAAGCTATACAGGGTCA |
| TWIST1 Forward | GGGCCGGAGACCTAGATG |
| TWIST1 Reverse | TTTCCAAGAAAATCTTTGGCATA |
| KLHL29 Forward | GAGGACCGACTTCCCTCTC |
| KLHL29 Reverse | CCCAGGGTGTGGACTGATTG |
| CACNA1C Forward | TTCATGTTTGCCTGCCATCG |
| CACNA1C Reverse | GAGGAAGTCAAAAACATTCCAGG |
| β-actin Forward | GCACCACACCTTCTACAATG |
| β-actin Reverse | TGCTTGCTGATCCACATCTG |
| miR-92a-3p Forward | GGGGCAGTTATTGCACTTGTC |
| miR-92a-3p Reverse | CCAGT GCAGGGTCCGAGGTA |
| U6 Forward | TCCGGGTGATGCTTTTCCTAG |
| U6 Reverse | CGCTTCACGAATTTGCGTGTCAT |

### 2.10. Cell transfection

MiR-92a-3p mimic (M, miR10000092-1-5), mimic control (MC, miR1N0000001-1-5), inhibitor (I, miR20000092-1-5) and inhibitor control (IC, miR2N0000001-1-5) were purchased from RIBOBIO (www.ribobio.com, Guangzhou, China). KLHL29 expression was up-regulated by pcDNA3.1 transfection, and the transfection of empty pcDNA3.1 vector was used as negative control. Vectors used in this assay for KLHL29 overexpression were purchased from GenePharma (www.genepharma.com, Shanghai, China).

The transfection was completed with the help of nanofecter transfection reagent (164410-01, Procell, China). After 24 h of transfection, the results of transfection were observed by qRT-PCR.

### 2.11. Bioinformatics analysis

The GEO database GSE82107 was analyzed by GEO2R to compare the gene expression profiles of synovial tissues collected from normal and OA patients, and the intersection results with the downstream target genes of miR-92a-3p predicted by Targetscan (http://www.targetscan.org/vert_72/) were obtained (RBM47, SCN8A, CAND1, TWIST1, KLHL29, ATRX, STX17, CACNA1C, CEP3500). TWIST1, KLHL29 and CACNA1C expressions were up-regulated in OA.

### 2.12. Dual-luciferase reporter assay

The binding site between miR-92a-3p and KLHL29 was predicted by Targetscan. The wild type (WT) sequence (CGCUCGCUCUGCCAGGUGCAAUA) and mutant (MUT) sequence (CGCUCGCUCUGCCAGGUGGCAUA) of KLHL29 containing the binding site of miR-92a-3p or not were inserted into the psiCHECK-2 vector (TB329, Promega, USA), respectively. The recombinant vector and miR-92a-3p M/MC were co-transfected into cells for 24 h. Afterwards, the luciferase activity was observed on the Cellometer Auto 2000 Cell Viability Counter (Nexcelom, USA) using Dual Luciferase Reporter Gene Assay Kit (11402ES60, Yeasen, China).

### 2.13. Statistical analyses

Data were analyzed by Graph Prism v8.0 (Graphpad software, California, USA) and SPSS 20.0 (SPSS, Chicago, USA). Comparisons between groups were calculated by one-way analysis of variance and a Student's *t*-test. All results were expressed as mean ± standard deviation. $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. The characterization of OA synoviocytes as well as the regulatory effect of the co-culture of MF and OA synoviocytes towards the release of cytokines

The cultured OA synoviocytes were composed of spindle-shaped synovial fibroblasts and star-shaped synovial macrophages (Fig. 1A). See Fig. 1B for the co-culture system. Synoviocytes were seeded in the lower chamber, and MF was seeded in the upper chamber (Fig. 1B). We then evaluated the effects of co-culturing MF and OA synoviocytes on the secretion of inflammatory factors in the supernatant. The level of CXCL8/IL-8 in Syn. + MF group was similar to that in Syn. group (Fig. 1C, $p > 0.05$). However, in Syn. + MF group, the levels of CCL2/MCP-1, CCL3/MIP-1α and CCL5/RANTES were greatly reduced as compared to those in Syn. group (Fig. 1D–F, $p < 0.05$). Then, we detected the levels of MMP-9 and its specific inhibitor TIMP-1. The

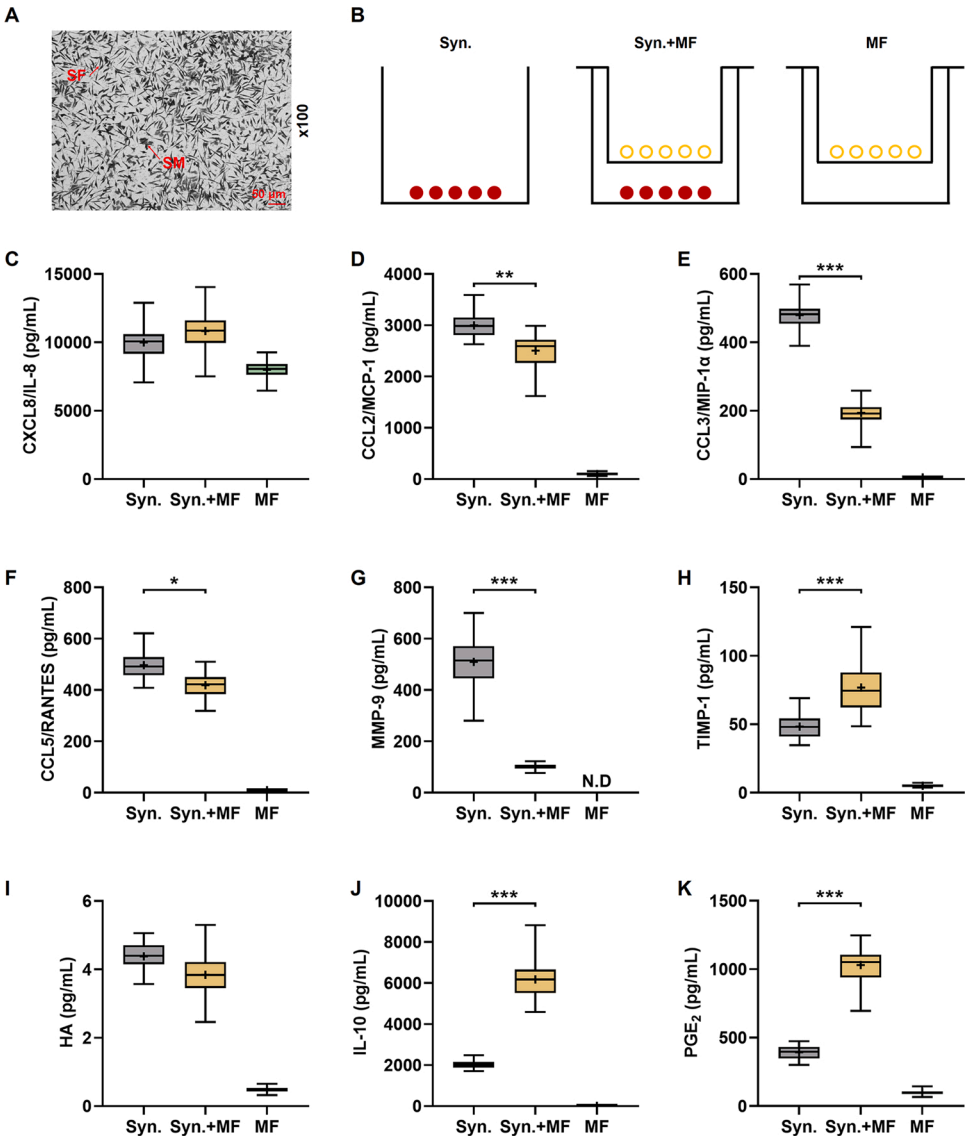


**Fig. 1.** Characterization of OA synoviocytes and effects of MF on cytokines released by OA synoviocytes. A, Morphology of OA synoviocytes (magnification, ×100). Scale bar = 50 μm. B, experimental design of co-culture system using a transmembrane with 0.4 μm pores. C—F, Inflammatory factors (CXCL8/IL-8, CCL2/MCP-1, CCL3/MIP-1α and CCL5/RANTES) released by synoviocytes and MF. G-H, MMP-9 and its specific inhibitor TIMP-1 released by synoviocytes and MF. I-J, anti-inflammatory factors (HA and IL-10) released by synoviocytes and MF. K, $PGE_2$ released by synoviocytes and MF. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to the Syn group. The experiments were constructed in three groups: Syn. group (normally cultured synoviocytes), Syn. + MF group (synoviocytes were co-cultured with MF) and MF group (mono-cultured MF). The experiments were performed repeated at least three times. Data are shown as boxes and whiskers. Lines inside the boxes represent the median, and whiskers represent the minimum and maximum values. Cultured synoviocytes were used as controls.
OA: osteoarthritis. MF: micro-fragmented adipose tissue. IL: Interleukin. MCP-1: monocyte chemotactic protein 1. MIP-1α: macrophage inflammatory protein 1α. RANTES: regulated upon activation normal T cell expressed and secreted factor. Syn: synoviocytes. $PGE_2$: Prostaglandin $E_2$. MMP-9: Matrix metalloproteinase 9. TIMP-1: Tissue Inhibitor of Metalloproteinases-1. HA: hyaluronic acid.

results showed that in Syn. + MF group, the secretion of MMP-9 was suppressed but the release of TIMP-1 was promoted (Fig. 1G-H, $p < 0.001$). At the same time, we also tested the levels of anti-inflammatory factors (HA and IL-10) and PGE2 by ELISA. The findings confirmed that in Syn. + MF group, the secretion of IL-10 and PGE2 was notably increased whereas the level of HA was not affected when compared to those in Syn. group (Fig. 1I–K, $p < 0.001$).

### 3.2. *TNF-α stimulation enhanced viability and impeded apoptosis of OA synoviocytes*

TNF-α was used to stimulate the inflammation *in vitro*. 5 and 10 ng/mL TNF-α promoted the cell viability and suppressed the cell apoptosis rate, whereas 1 ng/mL TNF-α exerted no significant effect (Fig. 2A–C, $p<0.05$). To further verify the effects of TNF-α on apoptosis, western blot was performed to detect the expressions of apoptosis-related proteins. The level of Bcl-2 was promoted by 1, 5, 10 ng/mL TNF-α, while the levels of Bax and cleaved caspase-3 were suppressed by 5 and 10 ng/mL TNF-α (Fig. 2D-E, $p<0.01$). Thus, we singled out 10 ng/mL TNF-α for subsequent experiments by virtue of its greatest impacts on cell viability and apoptosis.

### 3.3. *Co-culture of synoviocytes and MF reversed the inflammatory effects of TNF-α on OA synoviocytes*

Interestingly, the cell viability and apoptosis rate regulated by TNF-α were reversed by co-culture of synoviocytes and MF (Fig. 3A–C, $p<0.05$). Similarly, the upregulation of Bcl-2, CXCL8/IL-8, CCL2/MCP-1, CCL3/MIP-1α and CCL5/RANTES and the downregulation of Bax (except cleaved caspase-3) were overturned by co-culture of synoviocytes and MF (Fig. 3D–F, $p<0.01$).

### 3.4. *MiR-92a-3p I reversed the regulation of co-culturing synoviocytes and MF on OA synoviocytes*

Through qRT-PCR experiments, we found that the expression of miR-92a-3p was decreased in synoviocytes stimulated by TNF-α, while it returned to near the initial level after TNF-α-treated synoviocytes were co-cultured with MF (Fig. 4A, $p < 0.001$). We speculated that the biological regulation of synoviocytes by MF may be related to the regulation of miR-92a-3p expression. As such, we downregulated miR-92a-3p and tested its efficiency (Fig. 4B, $p < 0.001$). After downregulating miR-92a-3p in synoviocytes treated with TNF-α, the reduced expression of miR-92a-3p caused by co-culture of synoviocytes and MF was reversed (Fig. 4C, $p < 0.001$). Similarly, the downregulation of miR-92a-3p

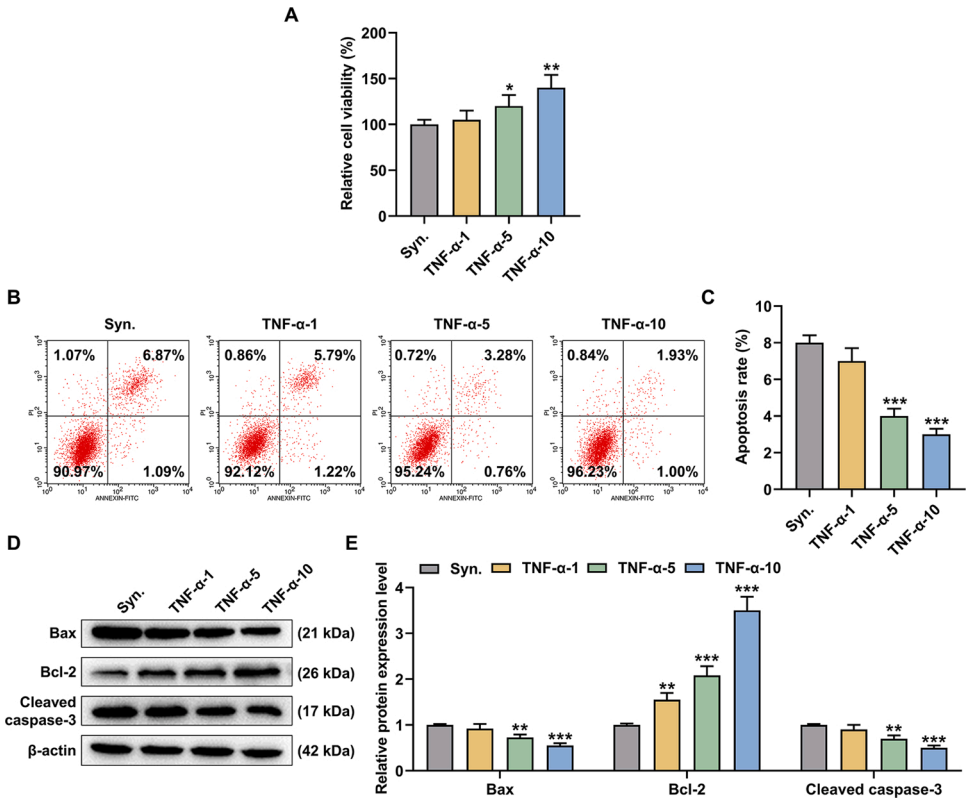


**Fig. 2.** $PGE_2$ affected the mRNA levels of inflammatory factors and TNF-α affected the cell viability, apoptosis and expression levels of apoptosis-related proteins.
A, the effects of TNF-α (1, 5, 10 ng/mL) on cell viability were detected by CCK-8. B-C, the effects of TNF-α (1, 5, 10 ng/mL) on cell apoptosis were measured by flow cytometry. D-E, western blot showed the effects of TNF-α (1, 5, 10 ng/mL) on levels of Bax, Bcl-2 and cleaved caspase-3. β-actin was used as an internal control. The experiments were established within four groups: Syn. group (normally cultured synoviocytes), TNF-α-1 group (synoviocytes were treated with 1 ng/mL TNF-α for 24 h), TNF-α-5 group (synoviocytes were treated with 5 ng/mL TNF-α for 24 h) and TNF-α-10 group (synoviocytes were treated with 10 ng/mL TNF-α for 24 h). Quantified values were mean ± standard deviation of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the Syn group.
$PGE_2$: Prostaglandin $E_2$. TNF-α: Tumor Necrosis Factor-α. IL: Interleukin. MCP-1: monocyte chemotactic protein 1. MIP-1α: macrophage inflammatory protein 1α. RANTES: regulated upon activation normal T cell expressed and secreted factor. qRT-PCR: quantitative reverse transcription polymerase chain reaction. Syn: synoviocytes. CCK-8: cell counting kit 8.

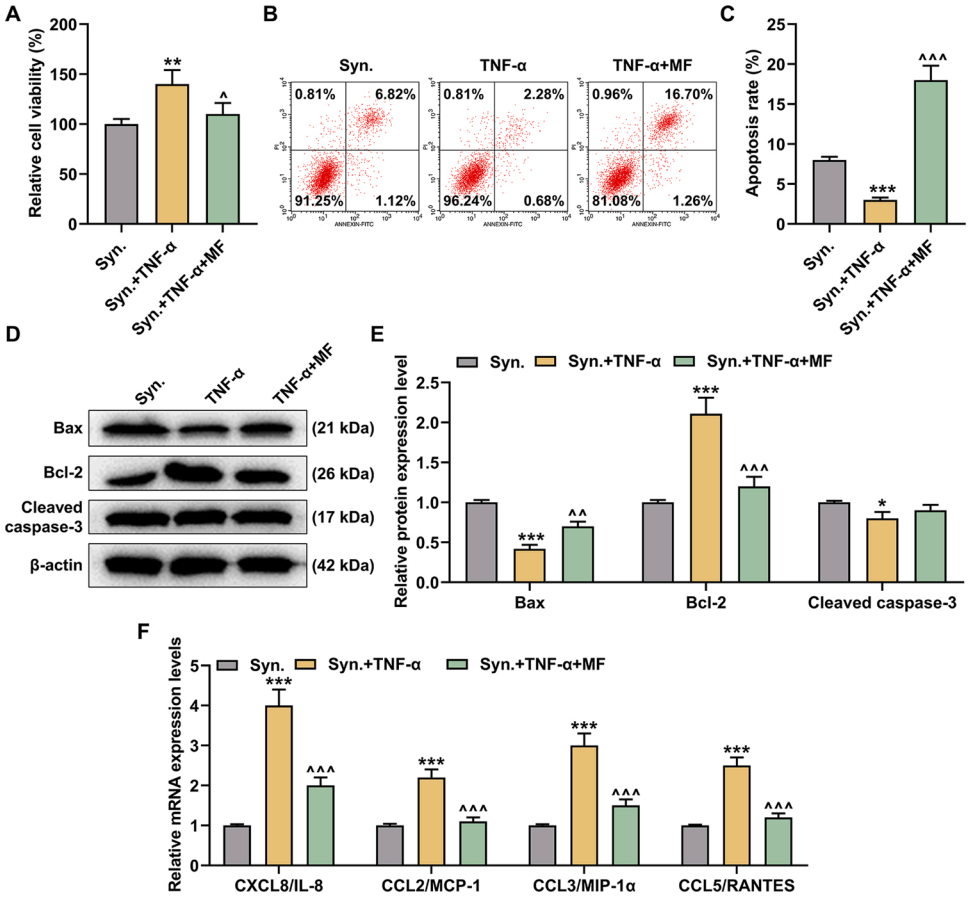


**Fig. 3.** Co-culture of synoviocytes and MF reversed the effects of TNF-α on cell viability, apoptosis and expressions of inflammatory factors.
A, CCK-8 was used to detect the cell viability in three groups. B-C, flow cytometry was performed to examine the apoptosis rate in three groups. D-E, western blot was used to determine the expressions of apoptosis-related proteins in three groups. β-actin was used as an internal control. F, qRT-PCR was performed to evaluate the inflammatory factors (CXCL8/IL-8, CCL2/MCP-1, CCL3/MIP-1α and CCL5/RANTES) expressions in three groups. β-actin was used as an internal control. The experiments were set up in three groups: Syn group, Syn + TNF-α group (synoviocytes were treated with 10 ng/mL TNF-α for 24 h), and Syn + TNF-α + MF group (synoviocytes were treated with 10 ng/mL TNF-α for 24 h and then co-cultured with MF). Quantified values were mean ± standard deviation of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the Syn group. $\hat{}P < 0.05$, $\hat{}\hat{}P < 0.01$, $\hat{}\hat{}\hat{}P < 0.001$ compared to the Syn. + TNF-α group.
TNF-α: Tumor Necrosis Factor-α. CCK-8: cell counting kit 8. MF: micro-fragmented adipose tissue. Syn: synoviocytes. qRT-PCR: quantitative reverse transcription polymerase chain reaction. IL: Interleukin. MCP-1: monocyte chemotactic protein 1. MIP-1α: macrophage inflammatory protein 1α. RANTES: regulated upon activation normal T cell expressed and secreted factor.

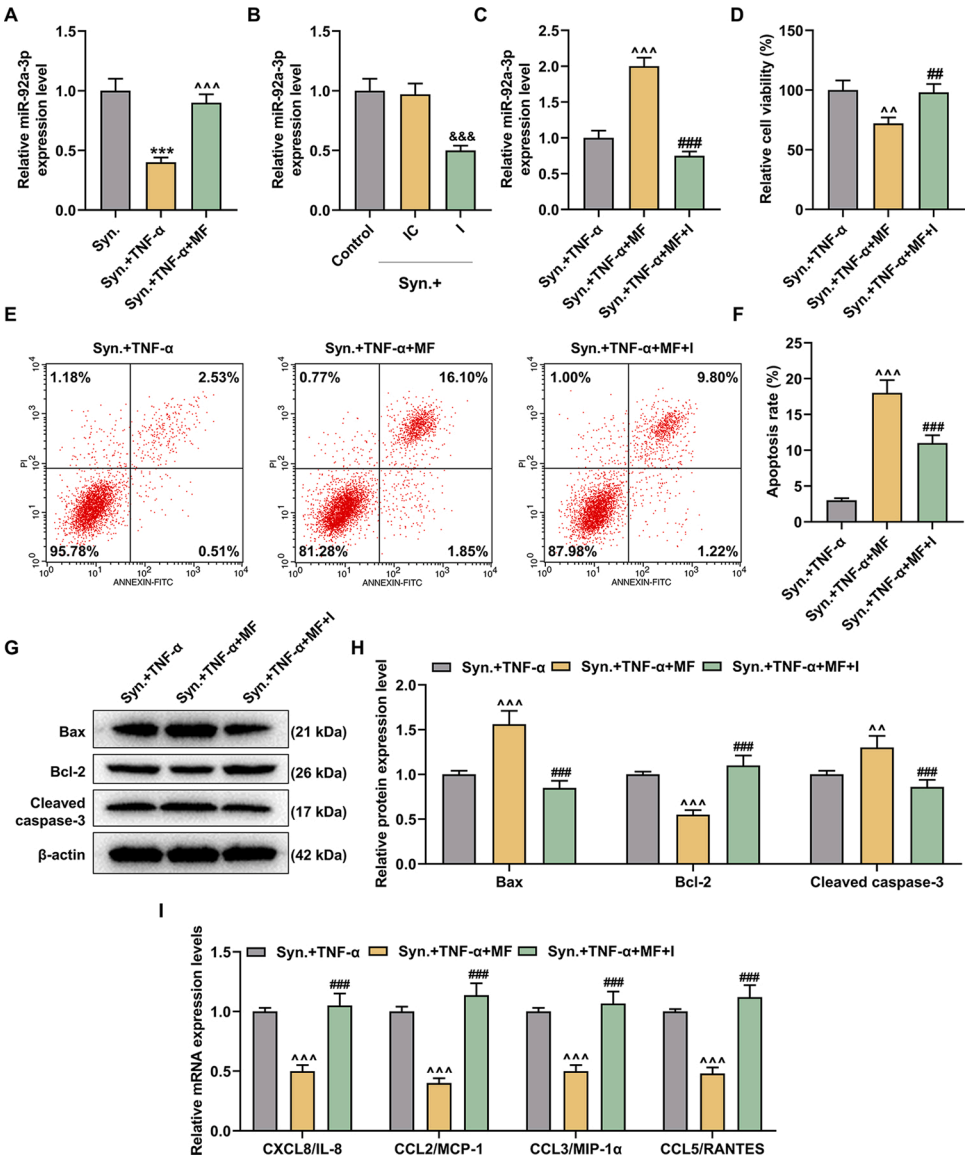


**Fig. 4.** MiR-92a-3p I reversed the regulation of co-culture of MF and TNF-α treated synoviocytes on cell viability, apoptosis and expressions of inflammatory factors.
A, qRT-PCR was performed to evaluate the expression of miR-92a-3p in three groups. U6 was used as an internal control. B, the transfection efficiency of miR-92a-3p was determined by qRT-PCR. U6 was used as an internal control. C, the expression of miR-92a-3p in three groups was determined by qRT-PCR. U6 was used as an internal control. D, CCK-8 detected the cell viability in three groups. E-F, flow cytometry examined the apoptosis rate in three groups. G-H, western blot was used to determine the apoptosis-related protein expressions in three groups. β-actin was used as an internal control. I, qRT-PCR was performed to evaluate the inflammatory factor (CXCL8/IL-8, CCL2/MCP-1, CCL3/MIP-1α and CCL5/RANTES) expressions in three groups. β-actin was used as an internal control. The experiments were grouped as follows: Syn. group (normally cultured synoviocytes), Syn + TNF-α group (synoviocytes were treated with 10 ng/mL TNF-α for 24 h), Syn + TNF-α + MF group (synoviocytes were treated with 10 ng/mL TNF-α for 24 h and then co-cultured with MF) and Syn + TNF-α + MF + I group (synoviocytes transfected with miR-92a-3p I were treated with 10 ng/mL TNF-α for 24 h and then co-cultured with MF). Quantified values were mean ± standard deviation of at least three independent experiments. $^{***}P < 0.001$ compared to the Syn group. $^{\&\&\&}P < 0.001$ compared to the IC group. $^{\wedge\wedge}P < 0.01$, $^{\wedge\wedge\wedge}P < 0.001$ compared to the Syn. + TNF-α group. $^{\#\#\#}P < 0.001$ compared to the Syn. + TNF-α +MF group.
MF: micro-fragmented adipose tissue. TNF-α: Tumor Necrosis Factor-α. CCK-8: cell counting kit 8. qRT-PCR: quantitative reverse transcription polymerase chain reaction. IL: Interleukin. MCP-1: monocyte chemotactic protein 1. MIP-1α: macrophage inflammatory protein 1α. RANTES: regulated upon activation normal T cell expressed and secreted factor. I: inhibitor. IC: inhibitor control.

overturned the effects resulted from the co-culture of TNF-α-treated synoviocytes with MF on cell viability, cell apoptosis, and expressions of apoptosis-related proteins and inflammatory factors (Fig. 4D–I, $p < 0.001$).

### 3.5. *KLHL29 was the target gene of miR-92a-3p*

Through bioinformatics analysis, we screened out 9 genes as candidate target genes of miR-92a-3p (Fig. 5A), among which only TWIST1, KLHL29 and CACNA1C levels were up-regulated in OA. Furthermore, qRT-PCR revealed that the mRNA expression of KLHL29 changed the most after miR-92a-3p was silenced (Fig. 5B, $p < 0.01$), so we intended to verify whether there was a targeting relationship between miR-92a-3p and KLHL29. Through the predicted binding sites (Fig. 5C), we constructed KLHL29-WT and KLHL29-MUT vectors. Subsequent dual-luciferase reporter assay validated that only the luciferase activity of KLHL29-WT was inhibited by miR-92a-3p M (Fig. 5D, $p < 0.001$). Not only that, the decreased KLHL29 level after the co-culture of TNF-α-treated synoviocytes with MF was restored after miR-92a-3p expression was downregulated (Fig. 5E-F, $p < 0.01$). These evidences indicated that KLHL29 was the target gene of miR-92a-3p. Additionally, we stated the relationship of KLHL29 with inflammation and apoptosis in synoviocytes (Supplementary Fig. 1). The results manifested that KLHL29 overexpression markedly reversed the inhibitory effect of MF on the inflammation and apoptosis of TNF-α-induced OA synoviocytes (Supplementary Fig. 1A–H).

## 4. Discussion

There is increasing evidence that cytokines and chemokines exhibit widespread effects on synovial inflammation of OA. Studies have reported that the expressions of chemokines IL-8/CXCL-8, CCL2/MCP-1, CCL3/MIP-1α, CCL5/RANTES are abnormally upregulated in the synovial fluid and synoviocytes of OA patients and these chemokines can recruit inflammatory cells into the inflammatory lesions of OA, thereby exacerbating the patient's condition (Belluzzi et al., 2019; Gómez-Aristizábal et al., 2019; Yuan et al., 2001; Zhao et al., 2020). MMP-9 participated in the degradation of extracellular matrix, while its specific inhibitor TIMP-1 regulated the degradation rate of extracellular matrix (ECM) slower than the update rate (Fotopoulos et al., 2012). It

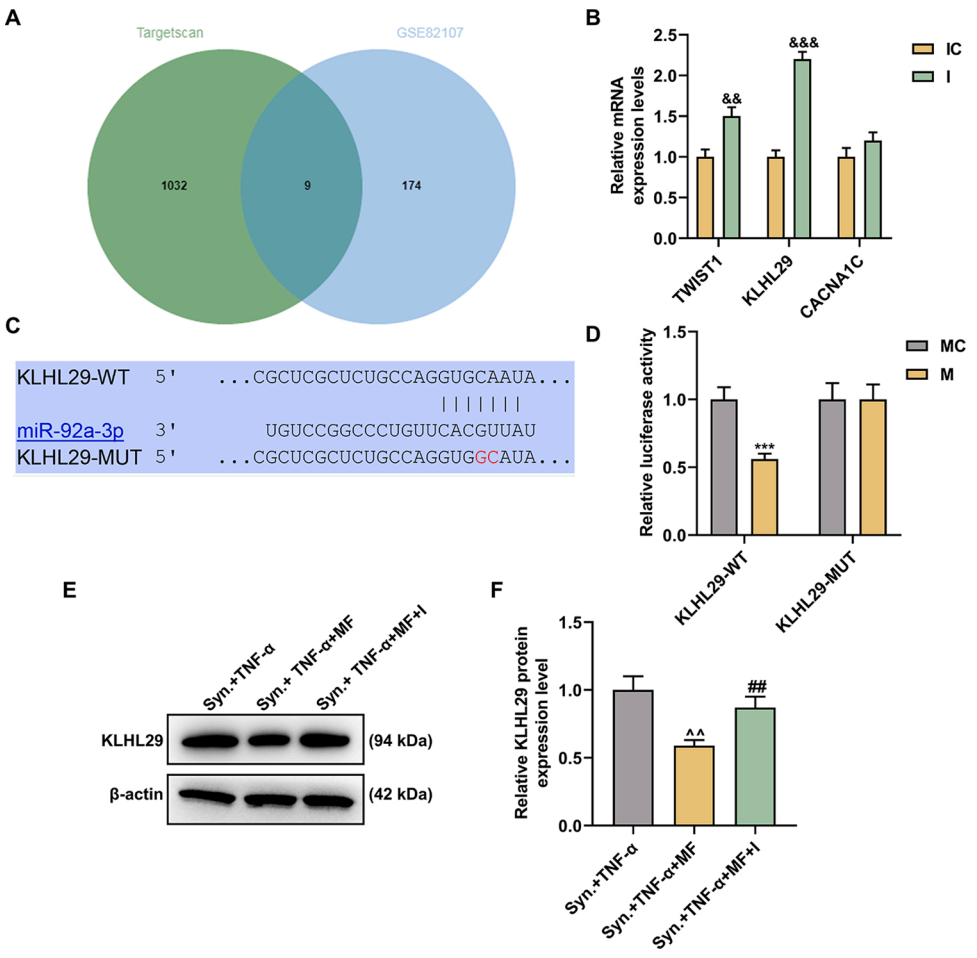


**Fig. 5.** KLHL29 was the target gene of miR-92a-5p.

A, Targetscan (http://www.targetscan.org/vert_72/) and GEO database GSE82107 were used to predict the target genes of miR-92a-5p. B, the expression levels of miR-92a-3p possible target genes TWIST1, KLHL29 and CACNA1C in synoviocytes were detected by qRT-PCR. β-actin was used as an internal control. C, the binding site between KLHL29 and miR-92a-3p was predicted by Targetscan. D, luciferase activity in synoviocytes co-transfected with KLHL29 WT/MUT and miR-92a-3p M/MC was determined by Dual-Luciferase Reporter Assay. E-F, the expression level of KLHL29 in three groups was determined by qRT-PCR. β-actin was used as an internal control. The experiments were grouped as follows: Syn + TNF-α group (synoviocytes were treated with 10 ng/mL TNF-α for 24 h), Syn + TNF-α + MF group (synoviocytes were treated with 10 ng/mL TNF-α for 24 h and then co-cultured with MF) and Syn + TNF-α + MF + I group (synoviocytes transfected with miR-92a-3p I were treated with 10 ng/mL TNF-α for 24 h and then co-cultured with MF). Quantified values were mean ± standard deviation of at least three independent experiments. $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$ compared to the IC group. $^{***}P < 0.001$ compared to the MC group. $^{\wedge\wedge}P < 0.01$ compared to the Syn. + TNF-α group. $^{\#\#}P < 0.01$ compared to the Syn. + TNF-α +MF group.

MF: micro-fragmented adipose tissue. TNF-α: Tumor Necrosis Factor-α. qRT-PCR: quantitative reverse transcription polymerase chain reaction. I: inhibitor. IC: inhibitor control. M: mimic. MC: mimic control.

was noted that MMP-9 was highly expressed in OA synovial fluid and took part in the pathogenesis of OA, which may be associated with the imbalance of MMP-9/TIMP-1 in OA patients (Naito et al., 1999). HA occupied a pivotal position in maintaining cartilage integrity and hindering the spread of bacterial toxins (Hemmati-Sadeghi et al., 2018). IL-10, as the main anti-inflammatory cytokine, reduced joint swelling, inhibited inflammatory cell infiltration, and improved cartilage degradation (Fernandes et al., 2020).

According to reports, MF can exert powerful anti-inflammatory potential in inflammatory diseases by regulating the secretion of inflammatory factors. A study has affirmed that the injection of autologous MF into a mouse model of sepsis signally alleviated the acute inflammatory response by suppressing the release of IL-6 and IL-1 (Bougle et al., 2018). Co-culture of MF and OA synoviocytes observably reduced the secretion of inflammatory factors (IL-6, CCL5/RANES, CCL2/MCP-1, CCL3/MIP-1α) (Paolella et al., 2019). In our study, we stimulated synoviocytes cultured *in vitro* with TNF-α to amplify the inflammatory cascade of OA. TNF-α not only stimulated the expressions of integrins and adhesion molecules on the surface of endothelial cells, promoted the aggregation of leukocytes, and induced joint inflammation, but also facilitated the release of other pro-inflammatory cytokines and synergistically strengthened the inflammatory response (Chisari et al., 2020; Fernandes et al., 2002). After TNF-α-treated synoviocytes were co-cultured with MF, the levels of CCL2/MCP-1, CCL3/MIP-1α, CCL5/RANTES and MMP-9 were dwindled, and those of TIMP-1 and $PGE_2$ were evidently promoted, implying that MF fulfilled a regulatory function on the cytokine secretion of OA synoviocytes, and this regulatory effect contributed to the inhibition of inflammatory response.

In addition to the complex cytokine network *in vivo*, the severity of synovitis was closely related to the insufficient apoptosis in OA synovial tissues. Therefore, it can effectively inhibit the development of OA by promoting the apoptosis of synoviocytes (Mao et al., 2019). Bcl-2, Bax and cleaved caspase-3 were apoptosis-related proteins. Both Bcl-2 and Bax participated in the mitochondrial pathway of apoptosis (Lopez and Tait, 2015). Cleaved caspase-3 was the activated form of caspase-3 which was the executor of caspase cascade involved in cell apoptosis (Porter and Janicke, 1999). We found that the level of anti-apoptosis protein Bcl-2 was suppressed while those of pro-apoptosis protein Bax and cleaved caspase-3 were promoted after TNF-α-treated synoviocytes were co-cultured with MF, indicating that co-culture of MF and synoviocytes promoted the apoptosis of synoviocytes.

We unraveled that the expression level of miR-92a-3p in activated synoviocytes was lower than the normal level, but MF stimulation restored the level of miR-92a-3p. This finding is consistent with the study of Guping Mao et al. who found that the expression of miR-92a-3p is reduced in OA (Mao et al., 2017b). MiR-92a-3p is related to cartilage formation, and Guping Mao et al. pointed out that miR-92a-3p inhibits cartilage degradation by down-regulating the expressions of ADAMTS-4 and ADAMTS-5 (Mao et al., 2017a). In addition, miR-92a-3p may also delay the progression of OA by inhibiting cell proliferation and inflammation. Debiao Xiang et al. found that after the treatment of curcumin and nicotine-curcumin in LPS-induced vascular smooth muscle cells, the up-regulation of miR-92a-3p in the cells may inhibit endothelial inflammation (Xiang et al., 2021). We verified that the target gene of miR-92a-3p is KLHL29, a member of the Kelch-like (KLHL) gene superfamily (Dhanoa et al., 2013). There are few reports about KLHL29 and there are no reports about whether KLHL29 is related to OA or inflammatory diseases. Our study disclosed that the expression

of KLHL29 in activated synoviocytes was regulated by MF and miR-92a-3p I, connoting that MF may exert an anti-OA effect through the miR-92a-3p/KLHL29 axis. But this conjecture has not been put into practice, so we will clarify the mechanism of KLHL29 in OA in future research.

On the whole, our study proved that MF regulated the release of inflammatory factors and apoptosis of OA synoviocytes by upregulating miR-92a-3p expression. Our study provides a new direction for the treatment of OA and a theoretical basis for the clinical application of MF in OA.

## Data availability

The data that has been used is confidential.
All Data will be made available on request.

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## Availability of data and materials

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

## Authors' contributions

Substantial contributions to conception and design: Zongting Shi.

Data acquisition, data analysis and interpretation: Jun He, Jian He, Yuan Xu.

Drafting the article or critically revising it for important intellectual content: Zongting Shi.

Final approval of the version to be published: Zongting Shi, Jun He, Jian He, Yuan Xu.

Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved: Zongting Shi, Jun He, Jian He, Yuan Xu.

## Declaration of Competing Interest

The authors report no declarations of interest.

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Micro-Fragmented Adipose Tissue Regulated the Biological Function of Rheumatoid Arthritis Synoviocytes by Promoting the Release of PGE2 through EP4 Receptor[2019KY254].

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.tice.2021.101716.

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