Mesenchymal Stem Cells Inhibit the Proliferation and Migration of Fibroblast-like Synoviocytes in Rheumatoid Arthritis via Exosome-mediated Delivery of miRNAs

Liangyu Mi

Shanxi Medical University

Na Li

Shanxi Medical University

Jinfang Gao

Shanxi Academy of Medical Sciences: Shanxi Bethune Hospital

Xinyue Peng

Shanxi Medical University

Na Zhang

Shanxi Academy of Medical Sciences: Shanxi Bethune Hospital

Rongxiu Huo

Shanxi Medical University

Ying Liu

Shanxi Academy of Medical Sciences: Shanxi Bethune Hospital

Maomao Fu

Taiyuan Central Hospital

Liyun Zhang

Shanxi Academy of Medical Sciences: Shanxi Bethune Hospital

Ke Xu (✉ zhaoxuke@hotmail.com)

Shanxi Academy of Medical Sciences: Shanxi Bethune Hospital

Research

Keywords: Rheumatoid arthritis, Fibroblast-like synoviocytes, Human umbilical cord mesenchymal stem cells-derived Exosomes, miRNA, Proliferation, Migration

Posted Date: October 20th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-965123/v1>

Abstract

Background

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by aggressive and symmetrical polyarthritis. Fibroblast-Like Synoviocytes (FLSs) play a central role in the pathogenesis of RA. The abnormal expression of miRNAs in RA FLSs mediates RA joint inflammation—synovial hyperplasia—and tissue destruction; MSC-Exos-derived miRNAs are a potential RA treatment strategy. This study aimed to investigate the hUCMSC-Exos can deliver miRNA to RA FLSs and affect their biological properties.

Methods

Primary hUCMSCs were isolated and cultured by tissue adherence method—and surface markers were identified by flow cytometry. We used differential centrifugation combined with ultrafiltration to obtain hUCMSC-Exos and identify them. The primary RA FLSs were isolated and cultured by trypsin digestion—then surface markers were identified by flow cytometry. We labeled the RNA of hUCMSC-Exos. hUCMSC-Exos was co-cultured with RA FLSs then the dyed RA FLSs were observed with a fluorescence microscope.

GV493 with siRNA target sequences that could knock down Ago2 transfected hUCMSCs. Real-time PCR and Western blot analysis Ago2 levels in hUCMSCs and transfected hUCMSCs to determine knockdown efficiency. Lentivirus GV493 with the target with the highest knockdown efficiency to transfect hUCMSCs—named hUCMSC^{KD3-Ago2}. Then hUCMSC-Exos—hUCMSC^{KD3-Ago2}-Exos—hUCMSC^{NC}-Exos were extracted by the differential centrifugal method combined ultrafiltration method—then were identified. The real-time cell analysis was used to detect the proliferation and migration of RA FLSs in different concentrations of hUCMSC-Exos. Finally—we used the optimal concentration of hUCMSC^{KD3-Ago2}-Exos and hUCMSC^{NC}-Exos to intervene in RA FLSs.

Results

After incubation with hUCMSC-Exos and RA FLSs—the hUCMSC-Exos accumulated in the blue nuclei of RA FLSs. hUCMSC^{NC}-Exos and hUCMSC^{KD-Ago2}-Exos inhibited the proliferation and migration of RA FLSs compared—particularly the hUCMSC^{NC}-Exos had the significant inhibitory effect.

Conclusions

hUCMSC-Exos RNA can be taken up by RA FLSs—and hUCMSC may affect the proliferation and migration of RA FLSs via exosome-mediated delivery of miRNA.

Highlights

- hUCMSCs can deliver hUCMSC-Exos to RA FLSs.

- Knockdown of Ago2 in hUCMSCs would reduce Ago2 in hUCMSC-Exos to affect miRNAs in hUCMSC-Exos.
- hUCMSC-Exos inhibits the proliferation and migration of RA FLSs.
- hUCMSC-Exos may affect RA FLSs by delivering miRNAs.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by the abnormal synovial hyperplasia of joints and the destruction of cartilage and bone. In recent years, the treatment strategies for RA have developed rapidly, but there is still a long way to go before the complete cure of RA. Traditional NSAIDs are restricted by a variety of safety issues, while traditional DMARDs have a slow effect and serious side effects. Although the glucocorticoids can quickly eliminate joint swelling and reduce pain, it cannot prevent joint damage and the development of lesions; moreover, it also has potentially severe side effects after long-term use[1]. Although biological agents and molecular targeted drugs have improved remission rates in RA, these drugs are expensive and target a particular step in the progression of RA. Mesenchymal stem cells (MSCs) have been reported as an interesting therapeutic cell candidate for the treatment of RA due to their immunomodulatory and differentiation effects. Unfortunately, it also has been found to cause abnormal differentiation and tumor formation[2]. Patients with RA have a low treatment compliance rate and are prone to relapse after drug withdrawal. In short, none of the existing treatment methods for RA can enable patients to achieve long-term remission without drugs, and may even progress to persistent inflammation and progressive disability. Fibroblast-like synoviocytes (FLSs) are an important part of the synovial membrane, which play a significant role in the process of local synovial hyperplasia, inflammation, and cartilage degradation in RA patients[3]. MicroRNAs (miRNAs), composed of 19-23 nucleotides, are evolutionarily conserved single-stranded non-coding RNAs, which could negatively regulate protein expression by degrading target RNA or blocking its translation[4]. Existing studies have shown that miRNA in RA FLSs may participate in the pathological process of RA by regulating Wnt signaling pathway, NF- κ B pathway, and DNA methylation[5]. Mesenchymal stem cell-derived exosomes (MSC-Exos) are common membrane-bound nanovesicles with a size of about 30-150nm derived from mesenchymal stem cells through exocytosis, which could mimic the function of parental MSCs by transferring bioactive substances such as DNA, proteins/peptides, mRNA, miRNA, lipids, and organelles. MSC-Exos are more effective, less toxic, and more stable than the parental cells. Studies have shown that MSC-Exos can mediate intercellular communication and regulate different signaling pathways through miRNA transmission[6]. Thus, we have reason to believe the cell-free therapy based on MSC-Exos may be more effective in repairing the joint damage of RA, breaking through the limitations of existing treatments, and becoming a potential method for the treatment of RA. Excitingly, the miRNAs encapsulated in MSC-Exos transfer information to recipient cells[7]. Some studies characterized the mechanisms by which Exos, particularly through miRNAs, are involved in RA. The human MSC-Exos that overexpress miRNA-124a could inhibit cell proliferation, migration, and promote apoptosis in the FLSs line[8]. The bone marrow mesenchymal stem cell (BMMSC)-secreted exosomal miR-192-5p can delay the event of the inflammatory response in RA[9]. BMMSC-derived Exos

participate in the intercellular transfer of miR-320a and subsequently inhibit the progression of RA[10]. The DBA/1J mice BMMSC-derived miR-150-5p Exos decreased migration and invasion in RA FLSs [as well as reduced hind paw thickness] and the clinical arthritic scores in collagen-induced arthritis (CIA) mice[11]. Exos were extracted from normal MSCs with over-expressed miR-146a/miR-155 increased forkhead box P3 (Fox- P3) TGFβ IL-10 RORγt IL-17 and IL-6 gene expression in the CIA mice[12]. These researches confirmed the value of MSC-Exos to mediate the direct intracellular transfer of miRNA and showed the efficacy of this strategy in RA.

The miRNAs have been found extracellularly being encapsulated within Exos or associated with Argonaute (Ago)[13]. The mature miRNA is loaded into the RNA-induced silencing complex (RISC) which mediates the relationship between the interacting miRNA and its target mRNA molecule. The Ago protein is part of RISC and mediates the cleavage of target mRNA. Particularly Ago2 has splicing activity and can cut mRNA. Post-transcriptional Ago2 regulates miRNA expression abundance. Ago2 binds with miRNAs to form the RISC which can associate with multivesicular bodies which produce Exos after fusion with the plasma membrane[14]. In short Ago2 promotes miRNA maturation prevents miRNA degradation and controls the sorting of miRNA. Regulation of Ago2 could affect miRNA maturation.

In the current study We tested the uptake of hUCMSC-Exos by RA FLSs and knocked down Ago2 in hUCMSC to reduce the content of Ago2 in hUCMSC-Exos which is in order to the expression of miRNA in hUCMSC-Exos then analyzed the effect of miRNA in hUCMSC-Exos on RA FLSs. In summary we clarified that miRNA delivered by hUCMSC-Exos may affect the proliferation and migration of RA FLSs.

Ultimately the results provide a theoretical foundation for the application of hUCMSC-secreted exosomal miRNA in RA treatment.

Materials And Methods

Ethics Statement

Informed consent agreement forms were signed and collected from all participants before enrollment into our study. The research followed the tenets of the Declaration of Helsinki and was approved by the Ethical Committee of Shanxi Bethune Hospital (ethical approval code:2018LL007).

Sample Collection

The specimens of this study were obtained from patients in the Department of Orthopedics at Shanxi Bethune Hospital and the Second Hospital of Shanxi Medical University from March 2015 to March 2021.

The synovial tissues taken from arthroplasty or synovectomy were from patients with RA. All patients fulfilled the diagnosis of the American College of Rheumatology for RA.

Isolation and Culture of RA FLSs

The synovial tissues were collected cut under sterile conditions detached with trypsin-ethylenediaminetetraacetic acid (0. 25%EDTA Solarbio Beijing China) at 37°C for 1 h and centrifuged.

The cells were then collected and cultured in Dulbecco's modified Eagle's medium (DMEM)(Gibco®USA) containing 10% fetal bovine serum (FBS)(Gibco®USA). Cell passages were conducted every 3-4 days followed by 24-h adherent growth of cells at 37°C with 5% CO₂. The cells were detached with 0.25% EDTA and passaged after reached 85% confluence. The RA-FLSs at passage 3 to 5 were utilized for the following experiments.

Isolation and Culture of hUCMSCs

We stripped the umbilical vein and artery of the umbilical cord (the Department of Obstetrics and Gynecology of Shanxi Bethune Hospital supplied) of the healthy term neonates. The remaining tissues were cut to 0.5mm³ then inoculated in petri dishes. After the tissues were attached to the wall we added serum-free medium (Excell Bio®Chain). The cells were fused to 80% they were digested by trypsin passed 1:3. The p3-p5 generation hUCMSCs in a good growth state were used in the experiment.

Flow cytometry analysis

RA FLSs cell suspension (1×10^5 cells/mL) and hUCMSCs cell suspension (1×10^5 cells/mL) were used for flow cytometry (FCM) (ACEA®USA) analysis to detect cell surface antigen phenotype. RA FLSs were incubated on ice for 1h with anti-rat antibodies (BioLegend®USA) conjugated with fluorescein isothiocyanate (FITC) Allophycocyanin (APC) or phycoerythrin (PE). Then FCM evaluated and analyzed CDH 11 PDPN CD68 (Abcam®USA). The hUCMSCs were incubated with anti-human monoclonal antibodies (BioLegend®USA) conjugated with FITC or PE. The FCM evaluated and analyzed markers of hUCMSCs (CD105 CD73 and CD90) endothelial cells (CD31 and CD34) and hematopoietic cells (CD45) (Biolengd®USA).

Culturing of Hela cells

Hela cells supplied by the Chinese Academy of Sciences as Western blot positive reference cells were cultured at 37 °C in the incubator containing CO₂ with a volume fraction of 5% and saturated humidity. The liquid was changed once every two days. When cells fused to 80% they were passed at a ratio of 1:5.

Transfection of hUCMSCs with Lentivirus GV493

hUCMSCs suspension was inoculated in 96-well culture plates (5×10^4 cells/well) to ensure that the cell fusion rate was about 20% after 24 hours. Experimental groupings dosage of GV493 and transfection conditions are shown in Table 1. The cell culture medium was changed 16 hours after transfection. The fluorescence expression was observed under the fluorescence microscope (Olympus®Japan) after 72h.

Transfection of hUCMSCs with lentivirus GV493 with or without siRNA target

Because exosomal miRNA is associated with Ago2, we had designed three different siRNA targets that can knock down Ago2, then respectively loaded them on GV493 to transfect hUCMSCs to knock down hUCMSCs Ago2 and exosomal miRNA. The hUCMSCs suspension was inoculated in a 6-well plate (1×10^5 cells/well) whose fusion rate was about 20% after being cultured for 24 hours. hUCMSCs were transfected by GV493 according to the previously established transfection conditions. According to the different types of lentiviruses transfected, the experiment was divided into 4 groups: hUCMSC^{KD1-Ago2} group: LVPSC85384-1 transfected hUCMSCs; hUCMSC^{KD2-Ago2} group: LVPSC85385-11 transfected hUCMSCs; hUCMSC^{KD3-Ago2} group: LVPSC85386-11 transfected hUCMSCs; NC group: the empty GV493 transfected hUCMSCs. The hUCMSCs in the KD group were named hUCMSC^{KD-Ago2}, which including hUCMSC^{KD1-Ago2}, hUCMSC^{KD2-Ago2} and hUCMSC^{KD3-Ago2}. At the same time, those in the NC group were named hUCMSC^{NC}. The informations of group and virus are shown in Table 2. After 16 hours of transfection, the cells in each well were collected and transferred to T25 culture flasks for continued cultivation. The fluorescence rate was the positive transfection rate, which was observed under a fluorescence microscope 72 hours after transfection.

Detect the levels of Ago2 in hUCMSCs, hUCMSC^{KD-Ago2} and hUCMSC^{NC}

Real-time PCR analysis

Total RNA was extracted and purified using Trizol reagent (Shanghai Pufei Biotech Co., Ltd.) according to the manufacturer's instructions and RNA was precipitated using isopropanol. The RNA quality determined by the spectrophotometer was OD260/OD280: 1.8-2.0 and the RNA concentration was 1000–2000 µg/mL. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control, whose primers were synthesized by Guangzhou RiboBio Co., Ltd. Moreover, their sequences were as follows: GAPDH—F5'-TGACTTCAACAGCGACACCCA-3', R5'-CACCTGTTGCTGTAGCCAAA-3'; Ago2—F5'-TCCACCTAGACCCGACTTT-3', R5'-GTTCCACGATTTCCCTGTT-3'. Reverse transcription of the extracted RNA was carried out according to the protocols of the M-MLV kit (Promega, USA). Quantitative real-time PCR was performed using PCR Kit (QIAGEN, USA) on Real-time PCR instrument (Roche, USA). With GAPDH used as the normalizing controls, ΔC_t indicates the gene expression level in cells, and $2^{-\Delta \Delta C_t}$ reflects relative quantification.

Isolation and identification of Exos

We cultured hUCMSCs, hUCMSC^{KD3-Ago2} and hUCMSC^{NC} with serum-free medium (Excell, China) and separately collected supernatant, then we used ultrafiltration combined with differential centrifugation to obtain the corresponding exosomes. The process is shown in Figure 1.

Exos were visualized using transmission electron microscopy (JEM-2010 F, JEOL, Japan). The particle size of Exos was determined by highly sensitive nanoparticle size analysis and Nanoparticle Tracking Analysis (NTA) using a Zetasizer NanoZS (Malvern Instruments, Malvern, UK). Exos protein concentrations were detected using a BCA Protein Assay kit (Solarbio, Beijing, China).

Western blot analysis

The RIPA lysate was used to extract proteins from Hela cells, hUCMSCs, hUCMSC^{KD-Ago2} and hUCMSC^{NC}. 40 µg samples were separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, USA) after electrophoresis. After incubation with primary antibodies Anti-Argonaute-2 antibody (Abcam, Cambridge, MA, USA) 1:1000 dilution in TBST with 5% BSA, the membranes were washed then incubated with goat anti-rabbit IgG-Horseradish peroxidase (Santa Cruz, USA) 1:2000 dilution secondary antibodies. The signals were visualized with ECL chemiluminescent solution (Thermo Fisher, USA).

We used western blot to analyze the Exos surface antigen phenotype and Ago2 level. UCMSC-Exos, hUCMSC^{KD3-Ago2}-Exos and hUCMSC^{NC}-Exos proteins were separated on 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (Solarbio, China) and transferred to PVDF membrane after electrophoresis. PVDF membrane was blocked in TBST with 5% skim milk for 1h. After incubation with primary antibodies against CD9 (Abcam, Cambridge, MA, USA) 1:1000 dilution, CD63 (Abcam, Cambridge, MA, USA) 1:500 dilution, TSG101 (sino biological, China) 1:500 dilution and Argonaute-2 (Abcam, Cambridge, MA, USA) 1:1000 dilution in TBST with 5% BSA, the membranes were washed four times with TBST (8 min each time) then incubated with horseradish peroxidase-conjugated goat anti-rabbit (CST, USA) 1:10000 dilution secondary antibodies. After the ECL method combined with X-ray imaging and we analyzed the Ago2 level.

Verification of the uptake of RNA in hUCMSC-Exos by RA FLSs

According to the instructions, we have configured RNA Select™ Green Fluorescent Cell Stain (Thermo Fisher, USA) working fluid, then the working fluid was added to the prepared hUCMSC-Exos suspension to label RNA. Exosome Spin Columns (Thermo Fisher, USA) were used to remove residual dye from hUCMSC-Exos.

We set up control groups and hUCMSC-Exos intervention groups. First, 9mm cell climbing sheets (NEST, China) were plated into 48-well plates and inoculated with RA FLSs (2×10^5 cells/well). After the cells were adherent, the medium was changed. Complete medium was still added into the control wells, and the UCMSC-Exos intervention wells were added with complete medium with 200 µg/mL RNA-labeled hUCMSC-Exos, then cells were incubated at 37°C in the dark for 24h. Next, the cells were washed with PBS, fixed with the fixative, and the nucleus were stained with Hoechst 33342 (Solarbio, China); Finally, we took out the cell climbing sheets and mounted them and fluorescence image were visualized with wide-field high-content image analysis system (PerkinElmer, USA).

Analysis of the effect of hUCMSC-Exos on the proliferation and migration of RA FLSs

hUCMSC-Exos suspension was used to interfere with RA FLSs (0 µg/mL, 10 µg/mL, 30 µg/mL, 60 µg/mL, 90 µg/mL, 120 µg/mL, 150 µg/mL, 180 µg/mL concentration); real-time Cell Analysis (RTCA) (ACEA, USA) was performed to detect Cell Index every 1 h to reflect the effect of hUCMSC-Exos on the

proliferation and migration of RA FLSs. The RA FLSs suspension was inoculated into wells of E-Plate 16(ACEAUSA)(3×10^3 cells/well). After the cells adhered, the cell supernatant was replaced with the complete medium containing hUCMSC-Exos at different concentrations, and then the cells were cultured for 30 hours. Similarly, RTCA was performed to detect the effect of hUCMSC-Exos on the migration of RA FLSs. The lower chambers of CIM-Plate 16(ACEAUSA) were filled with 15% FBS medium, and the upper chamber was added with serum-free RA FLSs suspension containing different concentrations of hUCMSC-Exos (serum-free RA FLSs suspension containing different concentrations of hUCMSC-Exos (2.5×10^4 cells/well)), then the cells were cultured for 48 hours.

Assessment of hUCMSC-Exos miRNA on the proliferation and migration of RA FLSs

We determined that the intervention concentration is 150ug/mL, which is the concentration that hUCMSC-Exos has the strongest effect on the proliferation of RA FLSs. RA FLSs were inoculated on E-Plate 16 (6×10^3 cells/well) for 48 hours. After the cells of each group adhered, we changed the culture conditions according to Table 3. RTCA detected Cell Index every 0.5h. RA FLSs were inoculated on CIM-Plate 16 (3×10^4 cells/well) for 46h hours, and RTCA detected Cell Index every 0.5h to reflect cells migration. We constructed the RA FLSs culture system for each group of cells according to Table 4.

Statistical analysis

SAS 9.4 is used for data processing and statistical analysis. Normally distributed data with homoscedasticity are expressed as mean $\pm$ standard deviation, while the Non-normally distributed data are expressed as the interval between the median and quartile, and the qualitative data rate was expressed by the ratio. Statistical analysis: repeated measure ANOVA was used for normality; Non-normality was compared by non-parametric tests based on rank. P values less than 0.05 were considered statistically significant.

Results

Isolation, culturing, and identification of RA FLSs

RA FLSs grew by adhering to the substratum and displayed a fibroblast-like morphology (Figure 2a). Flow cytometry analysis of RA FLSs surface antigen phenotype revealed that PDPN, CDH-11 were expressed, while CD68 were not. (Figure 2b).

Isolation, culturing, and identification of hUCMSCs

hUCMSCs grew by adhering to the substratum and displayed a long spindle-like morphology (Figure 3a). Flow cytometry analysis of hUCMSCs surface antigen phenotype revealed that CD105, CD73, CD90 were expressed, while CD45, CD34, CD31 were not (Figure 3b).

Assessment of hUCMSCs transfected by GV493

After GV493 was used to transfect hUCMSC under different conditions for 72 hours, the cells had normal growth morphology. GV493 is moderately difficult to transfect hUCMSCs, and HiTransG P can significantly increase the transfection rate. The 80% fluorescence rate is most suitable between the B and C groups (Figure 4a). In other words, the corresponding MOI is between 10-50. It follows that as few viruses as possible transfect cells without affecting the cell morphology, we determined MOI=20.

According to the most suitable transfection conditions that MOI=20 and add HiTransG P, we used GV493 with or without different targets transfected hUCMSCs. It showed that they successfully transfected hUCMSCs (Figure 4b).

The Ago2 levels in hUCMSCs, hUCMSC^{KD-Ago2} and hUCMSC^{NC}

Real-time PCR analysis

The larger the cell ΔCt , the lower the target gene level. When the cell $\Delta Ct \leq 12$, the gene level of the cell is high; when $\Delta Ct \geq 16$, the gene level in the cell is low. $\Delta Ct = Ct \text{ value}^{Ago2} - Ct \text{ value}^{GAPDH}$. The average value of ΔCt in hUCMSCs is 8.91, so Ago2 is highly expressed in hUCMSCs. Amplification curve and dissolution curve of GAPDH and Ago2 of hUCMSCs, hUCMSC^{NC} and hUCMSC^{KD-Ago2} are shown in Figure 5a-f. Quantitative PCR showed that compared with hUCMSC^{NC}, the Ago2 gene knockdown efficiency of hUCMSCs^{KD1-Ago2}, hUCMSCs^{KD2-Ago2}, hUCMSCs^{KD3-Ago2} were 51.4%, 10.1% and 70.7% (Figure 5g).

Western blot analysis

Western blot showing Ago2 expression in Hela cells (positive reference cells), which proves that the antibody is qualified. Ago2 also expressed in hUCMSCs (Figure 6a), hUCMSC^{KD-Ago2} (Figure 6b) and hUCMSC^{NC} (Figure 6b). The gray band values of hUCMSC^{NC} are normalized, hUCMSC^{KD1-Ago2}, hUCMSC^{KD2-Ago2} and hUCMSC^{KD3-Ago2} band gray value ratios respectively are 0.848847139, 0.4890497, 0.373544889.

All in all, the results of real-time PCR and Western blot showed that the Ago2 knockdown rate was the highest in the hUCMSC^{KD3-Ago2}, so the corresponding target LVPSC85386-11 was selected for the experiment.

Exos isolation and characterization

Transmission electron microscopy revealed that hUCMSC-Exos, hUCMSC^{KD3-Ago2}-Exos, hUCMSC^{NC}-Exos were oval phospholipid bilayer-bound structures with diameters of 30-150 nm (Figure 7a). NTA showed that the diameter distribution of hUCMSC-Exos was a single peak, the median particle diameter was 146 nm, and the concentration was 1.8×10^{11} Particles/mL (Figure 7b). Highly sensitive nanoparticle size analysis showed that the particle size distributions of hUCMSC-Exos, hUCMSC^{KD3-Ago2}-Exos, and hUCMSC^{NC}-Exos were single peaks, and their peak values respectively were 142 nm, 106 nm, 122 nm (Figure 7c). The protein concentrations of hUCMSC-Exos, hUCMSC^{KD3-Ago2}-Exos, and

hUCMSC^{NC-Exos} respectively were 1.384ug/ul, 1.133ug/ul, 1.038ug/ul. Western blot showing CD9, CD63 and TSG101 expression in hUCMSC-Exos, hUCMSC^{KD3-Ago2-Exos}, hUCMSC^{NC-Exos} (Figure 7d). The level of Ago2 in hUCMSC^{KD3-Ago2-Exos} is lower than that in hUCMSC^{NC-Exos} and hUCMSC-Exos (Figure 7e-f).

The uptake of RNA in hUCMSC-Exos by RA FLSs

After hUCMSC-Exos with green fluorescent-labeled RNA and RA FLSs were incubated for 24 hours, we saw that the blue-stained nucleus of RA FLSs were accumulated by green fluorescence. It indicates that RA FLSs can uptake the RNA in hUCMSC-Exos, but compared with the control group, the amount of cells in the experimental group is reduced (Figure 8a-b).

The effect of hUCMSC-Exos on the proliferation and migration of RA FLSs

After 6 hours of inoculation, all the RA FLSs adhered to the wall. At the same time, we added the complete medium containing hUCMSC-Exos, which caused a sharp bulge at the 6th hour of the curve. The cell index proliferation curve showed flattening after 25h. The 150ug/mL hUCMSC-Exos inhibited the proliferation of RA FLSs most strongly (Figure 9a).

The proliferation of RA FLSs without hUCMSC-Exos intervention was slow, and we considered that it is related to fewer cells. In the subsequent experiments, the number of cells were increased to 6×10^3 cells/well. The migration curve showed that 120ug/mL hUCMSC-Exos strongly inhibited the migration of RA FLSs (Figure 9b).

The effect of hUCMSC-Exos miRNA on the proliferation and migration of RA FLSs

The proliferation curves and migration curves of RA FLSs reflect that hUCMSC^{NC-Exos} and hUCMSC^{KD3-Ago2-Exos} inhibited the proliferation and migration of RA FLSs compared with the blank control group at the same time ($p < 0.01$, $p < 0.05$), particularly the hUCMSC^{NC-Exos} had the strongest inhibitory effect, which. The proliferation and migration of RA FLSs in each group were statistically different in the Cell Index at different time points ($p < 0.01$, $p < 0.05$), and there were statistical differences between each group as a whole ($p < 0.01$, $p < 0.05$) (Figure 10a-b). The proliferation and migration of RA FLSs at different time points in each group were expressed by cell index (Table 5a-b). We knocked down the Ago2 and also reduced the expression of miRNA in hUCMSC^{KD3-Ago2-Exos}, so the Ago2 of hUCMSC^{KD3-Ago2-Exos} is less than hUCMSC^{NC-Exos}. Those results indicate that miRNA in hUCMSC-Exos could affect the proliferation and migration of RA FLSs. It combines with that RA FLSs could take up the RNA of hUCMSC-Exos, which concludes hUCMSC-Exos may affect the proliferation and migration of RA FLSs by delivering miRNAs to RA FLSs.

Discussion

MSCs have high proliferation-immune regulation and multidirectional differentiation capabilities. they could inhibit the proliferation and differentiation of various immune cells-secretion of inflammatory factors and antibody production-and promote the repair of damaged tissues. It has become a new method to explore the treatment of RA. We have proved that BMMSCs can inhibit The CXCL10/CXCR3 chemotactic axis adjusts the ratio of RANKL/OPG-differentiates directly into chondrocytes-and improves the bone destruction of CIA[15].

MSCs were initially isolated from bone marrow and later found to be widely distributed in various tissues such as umbilical cord-fat-synovium-and gums. Compared with other sources-UCMSCs have great differentiation potential-strong proliferation ability-low immunogenicity-wide sources-convenient materials-and no moral and ethical restrictions. In CIA rats-hUCMSCs inhibited the proliferation of T lymphocytes and promoted T lymphocyte apoptosis-down-regulated RORyt mRNA and protein expression-reduced the proportion of Th17 cells-up-regulated Foxp3 mRNA and protein expression-and increased the proportion of spleen Treg cells. In addition-hUCMSCs down-regulated the expression of RORyt and Foxp3 in the joints-inhibited IL-17 and promoted the expression of TGF-B in serum-thereby improving arthritis-delaying radiological progress-and inhibiting synovial hyperplasia[16]. UCMSCs are most likely to become pluripotent stem cells with clinical application prospects. However-UCMSCs have some problems in the treatment of RA. The survival and biological activity of UCMSCs after entering the body is not easy to control. Because MSCs treatment inhibits anti-tumor immune response-there is a potential carcinogenic risk[17]. At the same time-intravenous infusion of UCMSCs may cause capillary bed blockage-ectopic bone formation-cartilage formation-and tumor formation risks. Cell therapy of MSCs shows low implantation rate and poor survival. They play a role in animal models through paracrine[18][19]-in other words-the protective effects of MSCs secretome depended on the presence of MSC-Exos[20].

Extracellular vesicles (EVs) released by MSCs in resting or stress state can mimic the immune regulation and tissue repair effects of MSCs-including Exos-microparticles (MPs) and apoptotic bodies[21]. Our previous research shows hUCMSC-EVs can improve CIA by simulating the immunomodulatory effects of MSCs-regulating the proliferation-apoptosis and differentiation of T lymphocytes-and regulating the levels of inflammatory factors related to T lymphocytes[22]. As the most important component-Exos contains rich biological information delivery media such as mRNA and miRNA-which can regulate the function-survival and homing of immune cells. Its immunomodulatory effect is stronger than MPs[23]-and it has better properties as a non-cellular therapy than MSCs. hUCMSC-Exos are expected to become a new strategy for the treatment of RA.

Synovial hyperplasia can lead to irreversible cartilage and bone damage-which is the main pathological feature of RA and the difficulty in treatment. The Synovial lining layer is consisted of macrophage-like synovial cells and FLSs. The FLSs of RA patients have obstacles in growth regulation mechanism-resulting in excessive proliferation of FLSs cells and showing "tumor-like growth"-which plays a central

role in joint inflammation and cartilage degradation during the progression of RA. FLSs express vimentin, CD45, CD55, CD90, CDH11, PDPN and other molecules on the surface. They can produce inflammatory factors and chemokines, chemoattract immune cells to promote immune response and delay the resolution of inflammation[24]. RA FLSs may be a potential target for RA treatment.

Varieties of studies have reported that miRNAs in FLS can participate in the pathogenesis of RA by regulating multiple signaling pathways. MiRNAs could participate in the occurrence and development of diseases by blocking mRNA translation and then inhibiting protein expression. The Wnt signaling pathway is the first pathway worth mentioning. In RA FLSs, miRNAs participate in the pathogenesis of RA by regulating the Wnt signaling pathway. miR-26 increases the production of pro-inflammatory cytokines and promotes the apoptosis of RA FLSs[25]; miR-375 inhibits the production of matrix metalloproteinases and fibronectin[26]; the upregulation of miR-663 promotes the proliferation of RA FLSs[27]; miR-323-3P positively regulates the Wnt pathway to affect RA FLSs [28]. MiR-152 ectopic expression indirectly induces negative regulators of the Wnt pathway to inhibit FLS proliferation. It can regulate the Wnt signaling pathway at the level of ligand-receptor and intracellular factors to regulate a variety of proteins that promote the migration and differentiation of RA FLSs[29]. In addition, the miRNA in RA FLSs can also participate in the pathological progress of RA by regulating the NF- κ B pathway. The up-regulated MiR-155 and miR-146a in RA FLSs inhibit the NF- κ B pathway to promote the activation of RA FLSs and promote osteoclastogenesis[30][31]. miR-27a reduces the levels of MMP2, MMP9, MMP13 and Rho family proteins in RA FLSs[31], thereby inhibiting the migration and invasion of RA FLSs, and blocking the NF- κ B pathway of RA FLSs. In RA FLSs, miR-21 acts as an activator of the NF- κ B pathway to promote NF- κ B nuclear translocation and promote the proliferation of RA FLSs [32]; miR-23b inhibits IL-17-induced activation of the NF- κ B pathway and expression of inflammatory factors to inhibit the pathogenesis of RA [33]. miR-10a participates in the NF- κ B-YY1-miR 10a pathway to promote the production of inflammatory factors and the proliferation and migration of RA FLSs [34]; miR-203 depends on the activity of NF- κ B to regulate the expression of IL-6 in RA FLS [35]; miR-10a is expressed in RA FLSs, and inhibits the production of pro-inflammatory cytokines and matrix metalloproteinases by inhibiting NF- κ B p65 induced by tumor necrosis factor- α ; miR-18a directly acts on tumor necrosis factor- α -induced protein 3 to inhibit NF- κ B pathway promotes the production of matrix-degrading enzymes and inflammatory mediators in RA FLS to promote the development of inflammation and cartilage destruction [36]; miR-19b positively regulates NF- κ B signaling to promote inflammatory activation of RA FLSs [37]; DNA methylation and miRNAs in RA FLSs. The interaction between miR-124a is involved in the pathogenesis of RA. The methylation of miR-124a in RA FLSs promotes the proliferation of RA FLS and the expression of tumor necrosis factor- α [38]; ectopic expression of miR-152 down-regulates DNA methyltransferase DNMT1 and activates Wnt Pathway[29]; high expression of miR-34a* enhances the apoptosis of RA FLSs mediated by Fas-L and TRAIL[39].

The regulation of RA FLS by hUCMSC-Exos through miRNA will become a potential strategy for RA treatment. The amount of miRNA in Exos is extremely low, sequence analysis showed that approximately 13% of the total RNA content of EVs from milk is miRNAs[40]. So we labeled the RNA of hUCMSC-Exos and then co-incubated it with RA FLSs, finally, found that RA FLSs could take up RNA in

hUCMSC-Exos. The RNA in hUCMSC-Exos may enter the RA FLSs directly through the surface protein and lipid ligands to directly activate the RA FLSs membrane surface receptors to generate signal complexes and activate the intracellular signaling pathway. It can also fuse with the plasma membrane of the RA FLSs or be endocytosed directly into the RA FLSs. The tracer methods for Exos include fluorescence imaging, magnetic resonance imaging, nuclear imaging, CT imaging, bioluminescence imaging, and photoacoustic imaging[41], which help us to monitor Exos biological behavior in real-time and promote the development and delivery of targeted Exos in the field of diagnosis and medicine.

In addition, the co-incubation of hUCMSC-Exos and RA FLSs affected the proliferation and migration of RA FLSs. The 150ug/ml hUCMSC-Exos strongly inhibited the proliferation of RA FLSs, and 120ug/ml hUCMSC-Exos strongly inhibited the migration of RA FLSs. MSC-Exos can transport various biologically active proteins and RNA (mRNA, miRNA, siRNA, etc.) to target cells to perform their biological functions. MSCs-derived exosomal miRNA-124a[8], miR-192-5p[9], miR-320a[10], miR-150-5p [11] and miR-146a/miR-155 [12] have been proven to be the treatments strategy for RA. In the current study, we knocked down Ago2 in hUCMSC, and then the content of Ago2 in hUCMSC-Exos decreased. Ago2 participates in the sorting of miRNA in Exos, so the knockdown of Ago2 reduces the miRNAs expression exported by hUCMSC-Exos[14]. We used ago2 knockdown and non-knockdown hUCMSC-Exos to interfere with RA FLSs, respectively, both of which inhibited the proliferation and migration of RA FLSs, especially the hUCMSC-Exos non-knockdown of ago2 had a stronger effect. The interfered miRNA in hUCMSC-Exos has a reduced effect on RA FLSs, indicating that miRNA in hUCMSC-Exos affects the proliferation and migration of RA FLSs. Combining with that, RA FLSs could uptake the RNA of hUCMSC-Exos, we clarified that miRNA delivered by hUCMSC-Exos might affect the proliferation and migration of RA FLSs. In the future, we will use live cells to dynamically track hUCMSC-Exos in real-time to visualize the dynamic cellular uptake of Exos to provide a scientific basis for the interaction mechanism between Exos and cells. Not only that, we will detect the changes of miRNAs in Exos and RA FLSs before and after Exos intervention in RA FLSs, and select more differentially expressed miRNAs to find more strategies for RA treatment.

Conclusions

hUCMSC-Exos may affect RA FLSs by delivering miRNAs. We traced that the RNA of hUCMSC-Exos was taken up by RA FLSs, and then knocked down the expression of hUCMSC-Exos Ago2, and confirmed that hUCMSC-Exos miRNA could inhibit the proliferation and migration of RA FLSs. Next, we will clarify the miRNA profile in RA FLSs, predict and anchor related therapeutic genes, and use MSC-Exos-derived miRNAs to open new strategies for RA targeted therapy.

Abbreviations

RA: Rheumatoid arthritis; FLSs: Fibroblast-Like Synoviocytes; MSCs: Mesenchymal stem cells; miRNAs: MicroRNAs; MSC-Exos: Mesenchymal stem cell-derived exosomes; CIA: collagen-induced arthritis; Fox-P3: forkhead box P3; Ago: Argonaute; RISC: RNA-induced silencing complex; DMEM: Dulbecco's modified

Eagle's medium; FBS:fetal bovine serum ;FCM:flow cytometry; FITC:fluorescein isothiocyanate; APC:Allophycocyanin;PE:phycoerythrin; GAPDH:Glyceraldehyde-3-phosphate dehydrogenase ; NTA:Nanoparticle Tracking Analysis ;FGF:Fibroblast growth factor;PVDF:polyvinylidene fluoride;RTCA:real-time Cell Analysis .

Declarations

Authors' contributions

KX directed the research. GLZ, NZ, YL and LYM designed the research. LYM and NL performed the experiments. analyzed and interpreted the data. and drafted the manuscript. LYZ participated in the design and revised the manuscript. LYM, XYP, RXH and MMF performed the experiments. All authors have read and approved the final manuscript.

Acknowledgements

The authors thank the Translational Medicine Research Center of Shanxi Medical University for the excellent technical assistance.

Funding

This work was supported by grants from the National Natural Science Foundation (81871292) and the Key Research and Development Projects of Shanxi Province (201803D31136).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. Ethics approval and consent to participate The study was approved by the ethical committee of Shanxi Bethune Hospital. All experimental procedures were performed according to the Institutional.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by the ethical committee of Shanxi Bethune Hospital. All experimental procedures were performed according to the Use Committee guidelines of Shanxi Medical University.

Consent for publication

Not applicable.

Competing interests

The authors have no competing interests.

References

1. Yu C, Li M, Duan X, Fang Y, Li Q, Wu R, Liu S, Wang Y, Wu Z, Shi X, Jiang Z, Wang Y, Hsieh ED, Jin S, Jiang N, Wang Q, Zhao Y, Tian X, Zeng X; and the co-authors of CREDIT. Chinese registry of rheumatoid arthritis (CREDIT): I. Introduction and prevalence of remission in Chinese patients with rheumatoid arthritis. *Clin Exp Rheumatol*. 2018 Sep-Oct;36(5):836-840. Epub 2018 Mar 21.
2. Luque-Campos N, Contreras-López RA, Jose Paredes-Martínez M, Torres MJ, Bahraoui S, Wei M, Espinoza F, Djouad F, Elizondo-Vega RJ, Luz-Crawford P. Mesenchymal Stem Cells Improve Rheumatoid Arthritis Progression by Controlling Memory T Cell Response. *Front Immunol*. 2019 Apr 16;10:798. doi: 10.3389/fimmu.2019.00798. PMID: 31040848; PMCID: PMC6477064.
3. Nygaard G, Firestein GS. Restoring synovial homeostasis in rheumatoid arthritis by targeting fibroblast-like synoviocytes. *Nat Rev Rheumatol*. 2020 Jun;16(6):316-333. doi: 10.1038/s41584-020-0413-5. Epub 2020 May 11. PMID: 32393826; PMCID: PMC7987137.
4. Wang L, Liu LZ, Jiang BH. Dysregulation of microRNAs in metal-induced angiogenesis and carcinogenesis. *Semin Cancer Biol*. 2021 Aug 21:S1044-579X(21)00221-2. doi: 10.1016/j.semcancer.2021.08.009. Epub ahead of print. PMID: 34428550.
5. Hong W, Zhang P, Wang X, Tu J, Wei W. The Effects of MicroRNAs on Key Signalling Pathways and Epigenetic Modification in Fibroblast-Like Synoviocytes of Rheumatoid Arthritis. *Mediators Inflamm*. 2018 May 10;2018:9013124. doi: 10.1155/2018/9013124. PMID: 29861659; PMCID: PMC5971246.
6. Nasirishargh A, Kumar P, Ramasubramanian L, Clark K, Hao D, Lazar SV, Wang A. Exosomal microRNAs from mesenchymal stem/stromal cells: Biology and applications in neuroprotection. *World J Stem Cells*. 2021 Jul 26;13(7):776-794. doi: 10.4252/wjsc.v13.i7.776. PMID: 34367477; PMCID: PMC8316862.
7. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol*. 2007 Jun;9(6):654-9. doi: 10.1038/ncb1596. Epub 2007 May 7. PMID: 17486113.
8. Meng HY, Chen LQ, Chen LH. The inhibition by human MSCs-derived miRNA-124a overexpression exosomes in the proliferation and migration of rheumatoid arthritis-related fibroblast-like synoviocyte cell. *BMC Musculoskelet Disord*. 2020 Mar 6;21(1):150. doi: 10.1186/s12891-020-3159-y. PMID: 32143603; PMCID: PMC7060528.
9. Zheng J, Zhu L, Lok In I, Chen Y, Jia N, Zhu W. Bone marrow-derived mesenchymal stem cells-secreted exosomal microRNA-192-5p delays inflammatory response in rheumatoid arthritis. *Int Immunopharmacol*. 2020 Jan;78:105985. doi: 10.1016/j.intimp.2019.105985. Epub 2019 Nov 24. PMID: 31776092.

10. Meng Q, Qiu B. Exosomal MicroRNA-320a Derived From Mesenchymal Stem Cells Regulates Rheumatoid Arthritis Fibroblast-Like Synoviocyte Activation by Suppressing CXCL9 Expression. *Front Physiol.* 2020 May 26;11:441. doi: 10. 3389/fphys. 2020. 00441. PMID: 32528301; PMCID: PMC7264418.
11. Chen Z, Wang H, Xia Y, Yan F, Lu Y. Therapeutic Potential of Mesenchymal Cell-Derived miRNA-150-5p-Expressing Exosomes in Rheumatoid Arthritis Mediated by the Modulation of MMP14 and VEGF. *J Immunol.* 2018 Oct 15;201(8):2472-2482. doi: 10. 4049/jimmunol. 1800304. Epub 2018 Sep 17. PMID: 30224512; PMCID: PMC6176104.
12. Tavasolian F, Hosseini AZ, Soudi S, Naderi M. miRNA-146a Improves Immunomodulatory Effects of MSC-derived Exosomes in Rheumatoid Arthritis. *Curr Gene Ther.* 2020;20(4):297-312. doi: 10. 2174/1566523220666200916120708. PMID: 32938348.
13. Guduric-Fuchs J, O'Connor A, Camp B, O'Neill CL, Medina RJ, Simpson DA. Selective extracellular vesicle-mediated export of an overlapping set of microRNAs from multiple cell types. *BMC Genomics.* 2012 Aug 1;13:357. doi: 10. 1186/1471-2164-13-357. PMID: 22849433; PMCID: PMC3532190.
14. McKenzie AJ, Hoshino D, Hong NH, Cha DJ, Franklin JL, Coffey RJ, Patton JG, Weaver AM. KRAS-MEK Signaling Controls Ago2 Sorting into Exosomes. *Cell Rep.* 2016 May 3;15(5):978-987. doi: 10. 1016/j. celrep. 2016. 03. 085. Epub 2016 Apr 21. PMID: 27117408; PMCID: PMC4857875.
15. Gao J, Zhang G, Xu K, Ma D, Ren L, Fan J, Hou J, Han J, Zhang L. Bone marrow mesenchymal stem cells improve bone erosion in collagen-induced arthritis by inhibiting osteoclasia-related factors and differentiating into chondrocytes. *Stem Cell Res Ther.* 2020 May 7;11(1):171. doi: 10. 1186/s13287-020-01684-w. PMID: 32381074; PMCID: PMC7203805.
16. Ma D, Xu K, Zhang G, Liu Y, Gao J, Tian M, Wei C, Li J, Zhang L. Immunomodulatory effect of human umbilical cord mesenchymal stem cells on T lymphocytes in rheumatoid arthritis. *Int Immunopharmacol.* 2019 Sep;74:105687. doi: 10. 1016/j. intimp. 2019. 105687. Epub 2019 Jul 8. PMID: 31295689.
17. Djouad F, Plence P, Bony C, Tropel P, Apparailly F, Sany J, Noël D, Jorgensen C. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. *Blood.* 2003 Nov 15;102(10):3837-44. doi: 10. 1182/blood-2003-04-1193. Epub 2003 Jul 24. PMID: 12881305.
18. Tang YL, Zhao Q, Qin X, Shen L, Cheng L, Ge J, Phillips MI. Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction. *Ann Thorac Surg.* 2005 Jul;80(1):229-36; discussion 236-7. doi: 10. 1016/j. athoracsur. 2005. 02. 072. PMID: 15975372.
19. Tögel F, Weiss K, Yang Y, Hu Z, Zhang P, Westenfelder C. Vasculotropic paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury. *Am J Physiol Renal Physiol.* 2007 May;292(5):F1626-35. doi: 10. 1152/ajprenal. 00339. 2006. Epub 2007 Jan 9. PMID: 17213465.

20. Lai RC, Arslan F, Lee MM, Sze NS, Choo A, Chen TS, Salto-Tellez M, Timmers L, Lee CN, El Oakley RM, Pasterkamp G, de Kleijn DP, Lim SK. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res.* 2010 May;4(3):214-22. doi: 10. 1016/j. scr. 2009. 12. 003. Epub 2010 Jan 4. PMID: 20138817.
21. Cosenza S, Ruiz M, Maumus M, Jorgensen C, Noël D. Pathogenic or Therapeutic Extracellular Vesicles in Rheumatic Diseases: Role of Mesenchymal Stem Cell-Derived Vesicles. *Int J Mol Sci.* 2017 Apr 22;18(4):889. doi: 10. 3390/ijms18040889. PMID: 28441721; PMCID: PMC5412468.
22. Xu K, Ma D, Zhang G, Gao J, Su Y, Liu S, Liu Y, Han J, Tian M, Wei C, Zhang L. Human umbilical cord mesenchymal stem cell-derived small extracellular vesicles ameliorate collagen-induced arthritis via immunomodulatory T lymphocytes. *Mol Immunol.* 2021 Jul;135:36-44. doi: 10. 1016/j. molimm. 2021. 04. 001. Epub 2021 Apr 12. PMID: 33857817.
23. Cosenza S, Toupet K, Maumus M, Luz-Crawford P, Blanc-Brude O, Jorgensen C, Noël D. Mesenchymal stem cells-derived exosomes are more immunosuppressive than microparticles in inflammatory arthritis. *Theranostics.* 2018 Feb 3;8(5):1399-1410. doi: 10. 7150/thno. 21072. PMID: 29507629; PMCID: PMC5835945.
24. Mi LY, Gao JF, Ma D, Zhang LY, Zhang GL, Xu K. Application of single-cell sequencing in autoimmune diseases. *Chin Med J (Engl).* 2020 Sep 1;134(4):495-497. doi: 10. 1097/CM9. 0000000000001050. PMID: 32889911; PMCID: PMC7909166.
25. Sun J, Yan P, Chen Y, Chen Y, Yang J, Xu G, Mao H, Qiu Y. MicroRNA-26b inhibits cell proliferation and cytokine secretion in human RASF cells via the Wnt/GSK-3 β / β -catenin pathway. *Diagn Pathol.* 2015 Jun 19;10:72. doi: 10. 1186/s13000-015-0309-x. PMID: 26088648; PMCID: PMC4472173.
26. Miao CG, Shi WJ, Xiong YY, Yu H, Zhang XL, Qin MS, Du CL, Song TW, Li J. miR-375 regulates the canonical Wnt pathway through FZD8 silencing in arthritis synovial fibroblasts. *Immunol Lett.* 2015 Mar;164(1):1-10. doi: 10. 1016/j. imlet. 2015. 01. 003. Epub 2015 Jan 22. PMID: 25619565.
27. Miao CG, Shi WJ, Xiong YY, Yu H, Zhang XL, Qin MS, Du CL, Song TW, Zhang B, Li J. MicroRNA-663 activates the canonical Wnt signaling through the adenomatous polyposis coli suppression. *Immunol Lett.* 2015 Jul;166(1):45-54. doi: 10. 1016/j. imlet. 2015. 05. 011. Epub 2015 May 28. PMID: 26028359.
28. Pandis I, Ospelt C, Karagianni N, Denis MC, Reczko M, Camps C, Hatzigeorgiou AG, Ragoussis J, Gay S, Kollias G. Identification of microRNA-221/222 and microRNA-323-3p association with rheumatoid arthritis via predictions using the human tumour necrosis factor transgenic mouse model. *Ann Rheum Dis.* 2012 Oct;71(10):1716-23. doi: 10. 1136/annrheumdis-2011-200803. Epub 2012 May 5. PMID: 22562984.
29. Miao CG, Yang YY, He X, Huang C, Huang Y, Qin D, Du CL, Li J. MicroRNA-152 modulates the canonical Wnt pathway activation by targeting DNA methyltransferase 1 in arthritic rat model. *Biochimie.* 2014 Nov;106:149-56. doi: 10. 1016/j. biochi. 2014. 08. 016. Epub 2014 Sep 4. PMID: 25194984.

30. Migita K, Iwanaga N, Izumi Y, Kawahara C, Kumagai K, Nakamura T, Koga T, Kawakami A. TNF- α -induced miR-155 regulates IL-6 signaling in rheumatoid synovial fibroblasts. *BMC Res Notes*. 2017 Aug 14;10(1):403. doi: 10. 1186/s13104-017-2715-5. PMID: 28807007; PMCID: PMC5556669.
31. Saferding V, Puchner A, Goncalves-Alves E, Hofmann M, Bonelli M, Brunner JS, Sahin E, Niederreiter B, Hayer S, Kiener HP, Einwallner E, Nehmar R, Carapito R, Georgel P, Koenders M, Boldin M, Schabbauer G, Kurowska-Stolarska M, Steiner G, Smolen JS, Redlich K, Blüml S. MicroRNA-146a governs fibroblast activation and joint pathology in arthritis. *J Autoimmun*. 2017 Aug;82:74-84. doi: 10. 1016/j. jaut. 2017. 05. 006. Epub 2017 May 22. PMID: 28545737; PMCID: PMC7233286.
32. Shi DL, Shi GR, Xie J, Du XZ, Yang H. MicroRNA-27a Inhibits Cell Migration and Invasion of Fibroblast-Like Synoviocytes by Targeting Follistatin-Like Protein 1 in Rheumatoid Arthritis. *Mol Cells*. 2016 Aug 31;39(8):611-8. doi: 10. 14348/molcells. 2016. 0103. Epub 2016 Aug 8. PMID: 27498552; PMCID: PMC4990753.
33. Zhu S, Pan W, Song X, Liu Y, Shao X, Tang Y, Liang D, He D, Wang H, Liu W, Shi Y, Harley JB, Shen N, Qian Y. The microRNA miR-23b suppresses IL-17-associated autoimmune inflammation by targeting TAB2, TAB3 and IKK- α . *Nat Med*. 2012 Jul;18(7):1077-86. doi: 10. 1038/nm. 2815. PMID: 22660635.
34. Mu N, Gu J, Huang T, Zhang C, Shu Z, Li M, Hao Q, Li W, Zhang W, Zhao J, Zhang Y, Huang L, Wang S, Jin X, Xue X, Zhang W, Zhang Y. A novel NF- κ B/YY1/microRNA-10a regulatory circuit in fibroblast-like synoviocytes regulates inflammation in rheumatoid arthritis. *Sci Rep*. 2016 Jan 29;6:20059. doi: 10. 1038/srep20059. PMID: 26821827; PMCID: PMC4731824.
35. Stanczyk J, Ospelt C, Karouzakis E, Filer A, Raza K, Kolling C, Gay R, Buckley CD, Tak PP, Gay S, Kyburz D. Altered expression of microRNA-203 in rheumatoid arthritis synovial fibroblasts and its role in fibroblast activation. *Arthritis Rheum*. 2011 Feb;63(2):373-81. doi: 10. 1002/art. 30115. PMID: 21279994; PMCID: PMC3116142.
36. Trenkmann M, Brock M, Gay RE, Michel BA, Gay S, Huber LC. Tumor necrosis factor α -induced microRNA-18a activates rheumatoid arthritis synovial fibroblasts through a feedback loop in NF- κ B signaling. *Arthritis Rheum*. 2013 Apr;65(4):916-27. doi: 10. 1002/art. 37834. PMID: 23280137.
37. Gantier MP, Stunden HJ, McCoy CE, Behlke MA, Wang D, Kaparakis-Liaskos M, Sarvestani ST, Yang YH, Xu D, Corr SC, Morand EF, Williams BR. A miR-19 regulon that controls NF- κ B signaling. *Nucleic Acids Res*. 2012 Sep;40(16):8048-58. doi: 10. 1093/nar/gks521. Epub 2012 Jun 7. PMID: 22684508; PMCID: PMC3439911.
38. Zhou Q, Long L, Zhou T, Tian J, Zhou B. Demethylation of MicroRNA-124a Genes Attenuated Proliferation of Rheumatoid Arthritis Derived Fibroblast-Like Synoviocytes and Synthesis of Tumor Necrosis Factor- α . *PLoS One*. 2016 Nov 8;11(11):e0164207. doi: 10. 1371/journal. pone. 0164207. PMID: 27824863; PMCID: PMC5100945.

39. Niederer F, Trenkmann M, Ospelt C, Karouzakis E, Neidhart M, Stanczyk J, Kolling C, Gay RE, Detmar M, Gay S, Jüngel A, Kyburz D. Down-regulation of microRNA-34a* in rheumatoid arthritis synovial fibroblasts promotes apoptosis resistance. *Arthritis Rheum.* 2012 Jun;64(6):1771-9. doi: 10.1002/art.34334. Epub 2011 Dec 12. PMID: 22161761.
40. Sun J, Aswath K, Schroeder SG, Lippolis JD, Reinhardt TA, Sonstegard TS. MicroRNA expression profiles of bovine milk exosomes in response to *Staphylococcus aureus* infection. *BMC Genomics.* 2015 Oct 16;16:806. doi: 10.1186/s12864-015-2044-9. PMID: 26475455; PMCID: PMC4609085.
41. Li YJ, Wu JY, Wang JM, Hu XB, Xiang DX. Emerging strategies for labeling and tracking of extracellular vesicles. *J Control Release.* 2020 Dec 10;328:141-159. doi: 10.1016/j.jconrel.2020.08.056. Epub 2020 Aug 31. PMID: 32882270.

Tables

Table 1 The groups and dosage of GV493 and infection conditions of hUCMSCs infected by GV493				
Infection Condition	A	B	C	Control
GV493 mount				
1×10 ⁸	Complete medium:90μL	Complete medium:86μL	Complete medium:86μL	Complete medium:100μL
MOI=100	GV493:10μL	HitransG A:4μL	HitransG P:4μL	
		GV493:10μL	GV493:10μL	
Group B	Complete medium:90μL	Complete medium:86μL	Complete medium:86μL	
5×10 ⁷	GV493:10μL	HitransG A:4μL	HitransG P:4μL	
MOI=50		GV493:10μL	GV493:10μL	
Group C	Complete medium:90μL	Complete medium:86μL	Complete medium:86μL	
1×10 ⁷	GV493:10μL	HitransG A:4μL	HitransG P:4μL	
MOI=10		GV493:10μL	GV493:10μL	
Group D	Complete medium:90μL	Complete medium:86μL	Complete medium:86μL	
1×10 ⁶	GV493:10μL	HitransG A:4μL	HitransG P:4μL	
MOI=1		GV493:10μL	GV493:10μL	

hUCMSC complete medium [Cyagen Biosciences USA] was used to cultivate hUCMSC. *HitransG P* [Genechem China] and *HitransG A* [Genechem China] are lentiviral infection enhancer. *GV493* was diluted with *hUCMSC basal medium* [Cyagen Biosciences USA] to different titers which corresponded to different multiplicity of infection [MOI].

Table 2 The groups and virus information of hUCMSCs infected by GV493 vector				
Group	virus name	Target sequence	Virus titer TU/mL	Virus dosage μL
KD1	LVPSC85384-1	gcACAGCCAGTAATCGAGTTT	2×10^9	2.00
KD2	LVPSC85385-11	cgGCAAGAAGAGATTAGCAAA	1×10^9	4.00
KD3	LVPSC85386-11	caATCAAATTACAGGCCAATT	1×10^9	4.00
NC	GW493		1×10^9	4.00

Table 3 The groups and culture conditions of RAFLSs in E-Plate 16 after RA FLSs adherence	
Groups (n=3)	Culture condition DMEM high sugar medium with 10% FBS
Blank control group	
hUCMSC ^{KD3-Ago2} -Exos group	150ug/m hUCMSC ^{KD3-Ago2} -Exos
hUCMSC ^{NC} -Exos group	150ug/mL hUCMSC ^{NC} -Exos

Table 4 The groups and culture system of RAFLSs in CIM-Plate 16

Group (n=3)	upper chamber Serum-free DMEM high sugar medium	lower chambers DMEM high sugar medium with 10% FBS
Blank control group		
hUCMSC ^{KD3-Ago2} -Exos group	150ug/mL hUCMSC ^{KD3-Ago2} -Exos	
hUCMSC ^{NC} -Exos group	150ug/mL hUCMSC ^{NC} -Exos	

Table 5a The proliferation of RA FLSs at different time points in each group

Group	Blank control group n=3	hUCMSC ^{KD3-Ago2} -Exos group n=3	hUCMSC ^{NC} -Exos group n=3
Time point			
10 h	1. 5394±0. 0724222	1. 37±0. 0753538	1. 3084333±0. 0346791
20 h	1. 5847667±0. 0686025	1. 3421667±0. 0810739	1. 2312333±0. 0228715
30 h	1. 5327±0. 0559024	1. 1817667±0. 0421836	1. 0848333±0. 0167804
40 h	1. 8286333±0. 0687184	1. 4050333±0. 0777613	1. 2751333±0. 0329749

Table 5b The migration of RA FLSs at different time points in each group

Group	Blank control group n=3	hUCMSC ^{KD3-Ago2} -Exos group n=3	hUCMSC ^{NC} -Exos group n=3
Time point			
10 h	1. 2837±0. 0145506	1. 2204667±0. 0461880	1. 0878000±0. 0775234
20 h	1. 098±0. 0401598	1. 0769±0. 0270217	0. 9862667±0. 0544596
30 h	1. 2325333±0. 0631646	1. 2112±0. 0409297	1. 1449667±0. 0442838
40 h	1. 3565±0. 0780123	1. 3427667±0. 041502	1. 2802333±0. 0581369

Figures

Figure 1

The supernatant of hUCMSCs was sequentially removed from dead cells and cell debris under different centrifugation conditions. After that the supernatant was concentrated with an ultrafiltration tube and filtered with 0.22µm filter membranes. Then the higher centrifugation conditions removed smaller debris. Finally ultra-high-speed centrifugation to pellet and wash exosomes.

Figure 2

Characteristics of RAFLSs. a The primary RAFLSs gradually adhere to the wall on the 3rd to 7th days and are fully grown and passable at about 18-25 days. They displayed a fibroblast-like morphology. When the growth density is $\geq 70\%$ they will appear "vortex". b RAFLSs express PDPN and CDH-11 positively on the surface and negatively express CD68

a

b

Figure 3

Characteristics of hUCMSCs. **a** hUCMSCs appeared on day 6 to 7 and grew by adhering to the substratum with a long spindle-like morphology . The growth density of HUCMSCs reached 80% in about 16-20 days. P2 hUCMSCs grow faster and can be subcultured in about 5 days. **b** FCM analysis of hUCMSCs surface antigen phenotype revealed that CD105 CD73 CD90 were expressed while CD45 CD34 CD31 were not .

Figure 4

a The morphology and proliferation of the transfected hUCMSCs were monitored under bright-field and dark field microscopy and they are normal. Adding HiTransG P to each group can enhance the transfection efficiency. The dark-field fluorescence rate of group A, group B, group C, and group D is about 100%, 90%, 60%, 20%. The best fluorescence rate is 80%, obviously. The best MOI is between 10-50. We finally choose MOI=20 and HiTransG P to transfect hUCMSCs. Expression of the green fluorescence

protein-GFP is observed as green colored hUCMSCs. b hUCMSCs morphology was monitored under bright-field microscopy as well as dark field microscopy . GV493 with or without siRNA targets could successfully transfected hUCMSCs-Expression of the GFP is observed as green colored hUCMSCs.

Figure 5

RT-PCR results of Ago2 in hUCMSCs-hUCMSCNC and hUCMSCKD-Ago2-a Amplification curves of GAPDH and Ago2 genes in hUCMSCs. b Dissolution curve of Ago2 in hUCMSCs. c Dissolution curve of

GAPDH in hUCMSCs. d Amplification curves of GAPDH and Ago2 in hUCMSCNC and hUCMSCKD-Ago2. e Dissolution curve of Ago2 in hUCMSCNC and hUCMSCKD-Ago2. f Dissolution curve of GAPDH in hUCMSCNC and hUCMSCKD-Ago2. g Knockdown efficiency of Ago2 in hUCMSCNC and hUCMSCKD-Ago2.

Figure 6

Ago2 in hUCMSCs, hUCMSCNC and hUCMSCKD-Ago2 were analyzed by Western blotting. a Expression of Ago2 in hUCMSCs. b Expression of Ago2 in hUCMSCNC and hUCMSCKD-Ago2. c Ago2 band gray value of hUCMSCNC and hUCMSCKD-Ago2.

Figure 7

The characteristics of hUCMSC-Exos, hUCMSCKD3-Ago2-Exos and hUCMSCNC-Exos . a Representative transmission electron microscopy (TEM) analysis from hUCMSCs, hUCMSCNC and hUCMSCKD3-Ago2.

b Nanoparticle tracking analysis (NTA) from hUCMSCs depicting size distribution patterns. c Highly sensitive nanoparticle size analysis from hUCMSC-Exos, hUCMSCKD-Ago2-Exos and hUCMSCNC-Exos showing particle size distributions. d hUCMSC-Exos, hUCMSCKD-Ago2-Exos and hUCMSCNC-Exo were analyzed by Western blotting using exosome markers. Representative images were shown for CD9, CD63 and TSG101. e Ago2 in hUCMSC-Exos, hUCMSCKD-Ago2-Exos and hUCMSCNC-Exos were analyzed by Western blotting. f Ago2 band gray value of hUCMSC-Exos, hUCMSCKD-Ago2-Exos and hUCMSCNC-Exos.

Figure 8

The uptake of RNA in hUCMSC-Exos by RAFLSs. a The nucleus of RA FLS stained by Hoechst 33342. b The blue-stained nucleus of RA FLSs were accumulated by green fluorescence, which is hUCMSC-Exos with green fluorescent-labeled RNA.

Figure 9

The effect of hUCMSC-Exos on the RA FLSs. **a** After different concentrations of hUCMSC-Exos interfere with RA FLSs the proliferation of RA FLSs is detected by real-time Cell Analysis (RTCA) and the 150ug/mL hUCMSC-Exos inhibited the proliferation of RAFLSs most strongly. **b** Cultured RA FLS in serum-free medium with different concentrations of hUCMSC-Exos in the upper chamber of CIM-Plate 16 added 15% FBS into the lower chamber of RTCA and RTCA detected the migration of RA FLS and the 120ug/mL hUCMSC-Exos inhibited the proliferation of RAFLSs most strongly.

a

b

Figure 10

The effect of hUCMSC-Exos miRNA on the proliferation and migration of RAFLSs. **a** The RA FLS proliferation curve showed that compared with the blank control both the hUCMSCKD3-Ago2-Exos and hUCMSCNC-Exos groups inhibited the proliferation of RAFLSs particularly hUCMSCNC-Exos had the strongest inhibitory effect. **b** The RA FLS migration curve showed that compared with the blank control both the hUCMSCKD3-Ago2-Exos and hUCMSCNC-Exos groups inhibited the migration of RAFLSs particularly hUCMSCNC-Exos had the strongest inhibitory effect.