Basic Study

Characterization of inflammatory factor-induced changes in mesenchymal stem cell exosomes and sequencing analysis of exosomal microRNAs

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Abstract

BACKGROUND
Treatments utilizing stems cells often require stem cells to be exposed to inflammatory environments, but the effects of such environments are unknown.

AIM
To examine the effects of inflammatory cytokines on the morphology and quantity of mesenchymal stem cell exosomes (MSCs-exo) as well as the differential expression of microRNAs (miRNAs) in the exosomes.

METHODS
MSCs were isolated from human umbilical tissue by enzymatic digestion. Exosomes were then collected after a 48-h incubation period in a serum-free medium with one of the following the inflammatory cytokines: None (control),...
vascular cell adhesion molecule-1 (VCAM-1), tumor necrosis factor (TNF) α, and interleukin (IL) 6. The morphology and quantity of each group of MSC exosomes were observed and measured. The miRNAs in MSCs-exo were sequenced. We compared the sequenced data with the miRBase and other non-coding databases in order to detect differentially expressed miRNAs and explore their target genes and regulatory mechanisms. In vitro tube formation assays and Western blot were performed in endothelial cells which were used to assess the angiogenic potential of MSCs-exo after inflammatory cytokine stimulation.

RESULTS

MSCs-exo were numerous, small, and regularly shaped in the VCAM-1 group. TNFα stimulated MSCs to secrete larger and irregular exosomes. IL6 led to a reduced quantity of MSCs-exo. Compared to the control group, the TNFα and IL6 groups had more downregulated differentially expressed miRNAs, particularly angiogenesis-related miRNAs. The angiogenic potential of MSCs-exo declined after IL6 stimulation.

CONCLUSION

TNFα and IL6 may influence the expression of miRNAs that down-regulate the PI3K-AKT, MAPK, and VEGF signaling pathways; particularly, IL6 significantly down-regulates the PI3K-AKT signaling pathway. Overall, inflammatory cytokines may lead to changes in exosomal miRNAs that abnormally impact cellular components, molecular function, and biological processes.

Key words: Mesenchymal stem cells; Exosomes; MiRNA; Inflammatory cytokines; Angiogenesis

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Core tip: The morphology and quantity of mesenchymal stem cell exosomes (MSCs-exo) are impacted in different inflammatory cytokine environments. Inflammatory cytokines impair the ability of MSCs-exo to promote angiogenesis. For instance, the tumor necrosis factor α and interleukin 6 groups exhibited decreased numbers of angiogenesis-related microRNAs (miRNAs), such as miR-196a-5p, miR-17-5p, miR-146b-5p, miR-21-3p, and miR-320. The same groups also had downregulated angiogenesis-related signaling pathways, such as PI3K-AKT and VEGF. Inflammatory cytokines may lead to changes in exosomal miRNAs that abnormally impact cellular components, molecular function, and biological processes, particularly angiogenesis-related miRNAs.

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INTRODUCTION

Stem cell transplantation has been developing rapidly and has resulted in breakthroughs for the treatment of various diseases. There is especially an interest in the transplantation of mesenchymal stem cells (MSCs), which are tissue-derived cells with self-renewal abilities. Their exosomes (MSCs-exo) not only contain the unique active components of all stem cells, but also are relatively more safe, are more chemically stable, and have the capacity for targeted delivery to biological pathways of interest[1-4].

Exosomes were first found in vitro in cultured sheep erythrocyte supernatant[5]. They are membranous vesicles with a diameter of 30-150 nm and a density of 1.10-1.18 g/mL. They are able to affect gene regulation by carrying and releasing various bioactive molecules such as microRNAs (miRNAs) and proteins, both of which can then function as paracrine signaling mediators impacting biological pathways...
relevant to disease processes\textsuperscript{6}. MiRNAs are non-coding RNAs 22-25 nucleotides in length\textsuperscript{7-10}. Exosomes are especially important in producing miRNAs that impact angiogenesis\textsuperscript{11}. There is promising research on using MSCs-exo to encourage wound healing and to treat inflammatory arthritis and ischemic diseases. However, in treating these diseases, MSCs are exposed to microenvironments filled with numerous inflammatory cytokines, such as vascular cell adhesion molecule-1 (VCAM-1), tumor necrosis factor (TNF) α, and interleukin (IL) 6. The effects of these inflammatory cytokines on the morphology and quantity of MSCs-exo and how these effects impact the production of miRNAs and downstream regulatory mechanisms are largely unknown. In this study, we analyzed the effects of VCAM-1, TNFα, and IL6 on the morphology and quantity of MSCs-exo, how these effects enhance differential expression of miRNAs, and how the target genes of these miRNAs and their associated regulatory mechanisms are regulated.

**MATERIALS AND METHODS**

**Cell culture**

Human umbilical mesenchymal stem cells were obtained from the Polywin Corporation (Guangzhou, China). The phenotypes of the MSCs were characterized by flow cytometric analysis of cell surface antigens, including tests for the cluster of differentiation (CD)29, CD34, CD44, CD73, and CD105. The MSCs were divided into four groups: Control group: MSCs (1.0 × 10\textsuperscript{5} cells/mL) were cultured in a serum-free DMEM/F12 (Sigma-Aldrich, United States) medium for 48 h; VCAM-1 group: MSCs (1.0 × 10\textsuperscript{5} cells/mL) were cultured in a serum-free DMEM/F12 (Sigma-Aldrich) medium, and VCAM-1 reagent (ADP5, R&D Systems, United States) was added to the medium at a concentration of 20 ng/mL for 48 h; TNFα group: MSCs (1.0 × 10\textsuperscript{5} cells/mL) were cultured in a serum-free DMEM/F12 (Sigma-Aldrich) medium, and TNFα reagent (T6674, Sigma-Aldrich) was added to the medium at a concentration of 20 ng/mL for 48 h; and IL6 group: MSCs (1.0 × 10\textsuperscript{5} cells/mL) were cultured in a serum-free DMEM/F12 (Sigma-Aldrich) medium, and IL6 reagent (200-06-20, PeproTech, United States) was added to the medium at a concentration 20 ng/mL for 48 h. The number, distribution, and morphology of cells in each group were observed under a microscope at 100× or 200× magnification for 48 h.

**Exosome isolation**

Exosomes were isolated from the culture supernatant by ultracentrifugation according to methods described previously\textsuperscript{12,13}. Briefly, the culture medium of each group was collected and centrifuged at 2000 × g for 10 min at 4 °C. The supernatant was then centrifuged at 10000 × g for 10 min at 4 °C. Next, the supernatant was passed through a 0.2-μm filter (Steradisc; Kurabo, Bio-Medical Department, Tokyo, Japan). The filtrate was ultracentrifuged at 100000 × g for 70 min at 4 °C (Type 70Ti ultracentrifuge; Beckman Coulter, Inc., Brea, CA, United States). The exosome-enriched fraction was next reconstituted in PBS for further studies. Characteristics and distributions of exosomes in each group were observed. The particle size and concentration of exosomes in each group were measured by nanoparticle tracking analysis.

**Differential miRNA analysis and target gene and regulatory signal pathway prediction**

The miRNAs of MSCs-exo were sequenced by BGISEQ-500 technology in each group. Sequenced data were compared with miRBase and other non-coding databases. Bioinformatics analysis pipeline steps for miRNA sequencing were: (1) Filtering small RNAs: 18-30 nt RNA segments were separated by polyacrylamide gel electrophoresis (PAGE); (2) 3’ adaptor ligation: A 5-adenylated, 3-blocked single-stranded DNA adapter was linked to the 3’ end of selected small RNAs from step 1; (3) Reverse primer annealing: the RT primer was added to the solution from step 2 and cross-linked to the 3’ adapter of the RNAs and to excess free 3’ adapter; (4) 5’ adaptor ligation: a 5’ adaptor was linked to the 5’ end of the product from step 3. The adaptor was attached to the end only, not to the 3’ adaptor or RT primer hybrid chain, thus greatly reducing self-ligation; (5) cDNA synthesis: The RT primers in step 3 were reverse extended to synthesize cDNA strands; (6) PCR amplification: High-fidelity polymerase was used to amplify cDNA, and cDNA with both 3’ and 5’ adaptors was enriched; (7) Library fragment selection: The PCR products of 100-120 bp were separated by PAGE to eliminate primer dimers and other byproducts; (8) Library
quantitative and pooling cyclization; (9) Eliminating the low-quality reads, adaptors and other contaminants to obtain clean reads; (10) Summarizing the length distribution of the clean tags, common, and specific sequences between samples; (11) Assigning the clean tags to different categories; (12) Predicting novel miRNAs; (13) Function annotation of known miRNAs; and (14) Comparing clean reads to the reference base group and other small RNA databases using AASRA software [14], except that Rfam was compared with cmsearch [15]. We used TPM [16] to standardize miRNA expression levels and predicted target genes using RNAhybrid [17], miRanda [18], and TargetScan [19].

Hierarchical clustering analysis showed differentially expressed miRNAs by functional heatmap. The P-values obtained from the differential gene expression tests were corrected by controlling the false discovery rate (FDR) [20] as more stringent criteria with smaller FDRs and bigger fold-change values can be used to identify differentially expressed miRNAs.

Gene ontology (GO) enrichment analysis was performed to identify all GO terms that are significantly enriched in a list of target genes of differentially expressed miRNAs, as well as the genes that correspond to specific biological functions. The hypergeometric test was then used to find significantly enriched GO terms based on this database (http://www.geneontology.org/). Pathway-based analyses were used to discover the biological functions of target genes using KEGG [21] (the major public pathway-related database).

**In vitro Matrigel tube formation assay**

HUVECs (4.0 × 10⁴, serum-starved overnight) were seeded in a 96-well plate, cultured in 5% CO₂ overnight, and then treated with PBS (control), control scrambled MSCs-exo, MSCs-exo⁴⁷⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻אפון {-⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻olygon

**Western blot analysis**

HUVECs (4.0 × 10⁴) were treated with PBS (control), control scrambled MSCs-exo, MSCs-exo⁴⁷⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻獴ון

**Statistical analysis**

The data from each group were collected and analyzed using SPSS 11.5 software (IBM SPSS China, Shanghai, china). Numerical data are presented as the mean ± SE; comparisons between groups were evaluated by Student’s t-test or ANOVA, with P < 0.05 considered significant.

**RESULTS**

**Phenotypic characterization of MSCs**

Cell purity (85% to 95%) was determined via flow cytometry. The cells were positive for mesenchymal cell markers such as CD29, CD44, CD73, and CD105 and negative for hematopoietic cell markers such as CD34 and HLA-DR (Figure 1).

**Effect of inflammatory cytokines on MSCs-exo**

The VCAM-1 group had small regularly shaped MSCs-exo. The TNFα group had large irregularly shaped MSCs-exo. The IL6 group had medium regularly shaped MSCs-exo (Figure 2).

The density of MSCs-exo was 7.42 × 10⁸/mL in the control group, 1.10 × 10⁹/mL in the VCAM-1 group, 7.37 × 10⁸/mL in the TNFα group, and 3.01 × 10⁸/mL in the IL6 group (Figure 3).

**Effect of differential miRNA expression, secondary to inflammatory cytokine exposure, on biological function**

Correlation analyses showed that the miRNA expression profiles in the IL6 group
Figure 1  Flow cytometric analysis of mesenchymal stem cell-related cell surface markers. High expression of positive mesenchymal cell markers (CD29, CD44, CD73, and CD105), and low expression of negative cell markers such as CD34 and HLA-DR were observed. $n = 3$ to 5; $P < 0.05$.

(0.583) and TNFα group (0.697) were more different from that of the control group than that in the VCAM1 group (0.985) (Figures 4 and 5). The top 10 miRNAs in each group are shown in Table 1. Many miRNAs, particularly some important angiogenesis-related miRNAs, were downregulated in the TNFα group and IL6 group compared to the control group (Tables 2-4 and Figure 6). Hierarchical clustering indicated that the expression levels of the majority of miRNAs in the IL6 group were downregulated compared with those of the control group (Figure 7). According to GO enrichment analysis, miRNAs in exosomes exposed to inflammatory cytokines, compared to controls, had a different regulatory effect on cellular components, molecular function, and biological processes (Figure 8). More specifically, pathway enrichment analysis showed that the target genes of the differentially expressed miRNAs, including those related to angiogenesis, differed among the four groups (Figures 9 and 10). The following angiogenesis-related pathways were more downregulated in the TNFα and IL6 groups than in the control group: The PI3K-AKT signaling pathway ($Q = 0.0978197212$, and 0.0581120875 in the TNFα group and IL6 group, respectively), the MAPK signaling pathway ($Q = 0.5775485$ and 0.9837761532 in the TNFα group and IL6 group, respectively), and the VEGF signaling pathway ($Q = 0.4082212190$ and 0.1711566 in the TNFα group and IL6 group, respectively) (Figures 10-13).
MSCs-exo promote endothelial cell angiogenesis

The ability of MSCs-exo to enhance tube formation was assessed using a Matrigel assay. MSCs-exo, on average, caused an increase in tube formation and branching ($P < 0.05$; Figure 14) compared to the untreated control group. The angiogenesis effect of MSCs-exo stimulated with TNFα and IL6 was lower than that cultured without TNFα or IL6. This finding confirmed that MSCs-exo can promote angiogenesis (Figure 14).

MSCs-exo stimulated with IL6 inhibit PI3K-AKT signaling pathway activation in endothelial cells

We observed a decline in the expression of phosphorylated AKT (pAKT) in endothelial cells after MSCs-exo were stimulated with IL6, although MSC-EV treatment had no effect on the expression of their unphosphorylated forms (Figure 15).

DISCUSSION

MSCs have been used to treat cardiovascular diseases (CVDs) and represent a promising cell-based therapy for regenerative medicine and the treatment of inflammatory and autoimmune diseases[22]. Their treatment efficacy hinges on their ability to alter disease-specific pathways via secreted miRNAs, so it is imperative to understand how disease environments, which often are inflammatory, can impact secreted miRNAs and thus potentially their treatment efficacy. MSCs-exosomes are used for treating CVDs such as acute myocardial infarction, stroke, pulmonary hypertension, and septic cardiomyopathy[23]. Biological properties of MSCs-exo have rendered them as a new strategy for wound regeneration and ischemic disease[24-26]. MSCs-exo exert an anti-inflammatory effect on T and B lymphocytes independently of MSCs priming. The potential therapeutic effects also were demonstrated in inflammatory arthritis[27].

Ischemic diseases, trauma, and immunological diseases are all accompanied by inflammatory reactions, and a large number of inflammatory cytokines including VCAM-1, TNFα, and IL6 are involved in the progression of these diseases. T cells are activated dependent on VCAM-1 interactions[28]. VCAM-1 plays a “backup” role in hASC contact-dependent immune suppression[29]. TNFα is a multifunctional cytokine
that acts as a central biological mediator for critical immune functions, including inflammation, infection, and antitumor responses\(^{30}\). IL6 plays an important role in the inflammatory response following hypoxic-ischemic encephalopathy\(^{31}\). Therefore, the biological properties of MSCs-exos and their therapeutic effects need to be studied together with the inflammatory factors. A large amount of evidence suggests that the effect of MSCs-exo therapeutics will be affected by the inflammatory environment with regard to most of CVDs and ischemic diseases\(^{32-40}\).

Some previous reports showed\(^{41}\) the effect of stimulation with cytokines interferon γ and TNFα on adipose MSCs (AMSCs). Pro-inflammatory stimuli could enhance the immunosuppressive functions of AMSC-derived exosomes. There was an increase in the expression of miRNAs (miR-34a-5p, miR21, and miR146a-5p) in exosomes produced by pre-activated AMSCs compared to those released by untreated cells.

The present study also found that the inflammatory cytokines VCAM-1, TNFα, and IL6 impact the size and morphology of MSCs-exo as well as the diversity of miRNAs they can produce, especially miRNAs impacting angiogenesis. According to GO enrichment analysis, miRNAs in exosomes exposed to inflammatory cytokines, compared to controls, had a different regulatory effect on cellular proliferation and differentiation, molecular signal transduction, immunosuppressive functions, angiogenesis and so on.

Some observed effects suggested that inflammatory cytokines impaired the ability of MSCs-exo to promote angiogenesis. For example, the TNFα and IL6 groups exhibited decreased numbers of angiogenesis-related miRNAs, such as miR-196a-5p, miR-17-5p, miR-146b-5p, miR-21-3p, and miR-320. The same groups also had downregulated angiogenesis-related signaling pathways, such as PI3K-AKT, MAPK, and VEGF. However, other effects suggested that inflammatory cytokines may promote the ability of MSCs-exo to encourage angiogenesis. Exosomes contained hsa-mir-4488, hsa-mir-671-5p, and hsa-mir-4446-3p after VCAM-1 stimulation, hsa-mir-4488, hsa-mir-671-5p, and hsa-mir-497-5p after TNFα stimulation, and hsa-mir-4488, hsa-mir-145-5p, and hsa-mir-1260a after IL6 stimulation, all of which promote angiogenesis. More specifically, hsa-mir-671-5p encourages NM_006500.2 to produce the downstream product VEGFb, which activates the VEGF pathway and thereby
Table 1  Differentially expressed miRNAs

| Control group | VCAM-1 group | In the TNFα group | In the IL6 group |
|---------------|--------------|-------------------|-----------------|
| sRNA id       | Count (16379497) | TPM | sRNA id | Count (23095609) | TPM | sRNA id | Count (20739574) | TPM |
| miR-21-5p     | 1544110       | 76933.99          | miR-21-5p       | 1640334         | 70885.38 | miR-21-5p | 1454726         | 70066.87 |
| miR-127-3p    | 194283        | 9724.54           | miR-127-3p      | 23095609        | 11250.19 | miR-127-3p | 408192          | 19681.79 |
| miR-199a-3p, miR-199b-3p | 139220 | 8499.65          | miR-199a-3p, miR-199b-3p | 153376 | 6727.51 | miR-127-3p | 321306 | 15492.41 |
| miR-100-5p    | 132865        | 8111.67           | miR-100-5p      | 140774          | 6095.27 | miR-100-5p | 271902 | 13110.3 |
| miR-222-3p    | 89564         | 5468.06           | miR-26a-5p      | 107800          | 4667.55 | miR-26a-5p | 140623 | 6780.42 |
| miR-26a-5p    | 77524         | 4732.99           | miR-34a-5p      | 86786           | 3757.68 | miR-223-2p | 129932 | 6264.93 |
| miR-34a-5p    | 76681         | 4681.52           | miR-431a        | 77297           | 3346.83 | miR-146a-5p | 128078 | 6175.54 |
| miR-181a-5p   | 67287         | 408             | miR-23a-3p      | 71930           | 3114.44 | miR-34a-5p | 119952 | 5783.73 |
| miR-411-5p    | 56606         | 3455.91           | miR-196a-5p     | 63939           | 2768.45 | miR-143-3p | 101398 | 4889.11 |
| miR-143-3p    | 49739         | 3036.66           | miR-181a-5p     | 62752           | 2717.05 | miR-26a-5p | 99628  | 4803.76 |

Count is the number of mapped tags, and the value in parentheses is the total number. Transcripts per kilobase million is the standardized expression value. VCAM-1: Vascular cell adhesion molecule-1; TNF: Tumor necrosis factor; IL6: Interleukin 6; TPM: Transcripts per kilobase million.

promotes angiogenesis. Hsa-miR-671-5p also encourages NM_001773.2 to produce CD34, which in turn activates the PI3K-AKT pathway, this promoting cell proliferation and angiogenesis. It is unclear what the ultimate results of these competing effects on angiogenesis would be.

Our study demonstrated that MSCs-exo perhaps induced HUVECs to form capillary-like structures in vitro. The effect of MSCs-exo in promoting angiogenesis would be reduced when the stem cells were subjected to TNFα and IL6 stimulation. Besides endothelial cell angiogenesis-related molecular expression, functional characteristics such as the PI3K-AKT signaling pathway may be down-regulated in MSCs-exo that were stimulated with IL6.

The major limitation of this study is that it was conducted in vitro. Further research needs to be conducted in vivo using an animal model that more closely mirrors the inflammatory environment to which MSCs are exposed when administered for treatment in humans.

In conclusion, inflammatory cytokines may lead to changes in exosomal miRNAs that abnormally impact cellular components, molecular function, and biological processes. Further in vivo research needs to be conducted to explore how the treatment efficacy of MSCs is impacted by these inflammatory-induced changes in exosomes and their miRNAs.
Table 2  Top 10 differentially expressed miRNAs in each group (Poisson distribution screening)

| miRNA id  | Count (control) | Count (VCAM-1) | TPM (control) | TPM (VCAM-1) | Log2 ratio (VCAM-1/control) | Up/down-regulation (VCAM-1/control) | P  | FDR  |
|---------|----------------|----------------|---------------|---------------|-----------------------------|-------------------------------------|----|------|
| miR-146a-5p | 20510          | 5173           | 1252.18       | 223.98        | -2.483                      | Down                                | 0  | 0    |
| miR-145-5p  | 3958           | 2391           | 278.27        | 103.53        | -1.426                      | Down                                | 0  | 0    |
| miR-225-3p   | 89564          | 48444          | 5468.06       | 2097.54       | -1.382                      | Down                                | 0  | 0    |
| miR-29a-3p  | 8451           | 5054           | 515.95        | 281.83        | -1.297                      | Down                                | 0  | 0    |
| miR-451a     | 2760           | 77297          | 168.5         | 3346.83       | 4.311                      | Up                                  | 0  | 0    |
| miR-496-5p   | 1839           | 475            | 112.27        | 20.57         | -2.448                      | Down                                | 0  | 0    |
| miR-1246      | 1812           | 6191           | 110.63        | 268.06        | 1.279                      | Up                                  | 0  | 0    |
| miR-181a-2-3p | 3400           | 2008           | 207.58        | 86.94         | -1.255                      | Down                                | 0  | 0    |
| miR-204-5p    | 3116           | 1757           | 190.24        | 76.08         | -1.322                      | Down                                | 0  | 0    |
| miR-507c      | 2              | 817            | 0.12          | 35.37         |                             | Up                                  | 0  | 0    |

| miRNA id  | Count (control) | Count (TNFα) | TPM (control) | TPM (TNFα) | Log2 ratio (TNFα/control) | Up/down-regulation (TNFα/control) | P  | FDR  |
|---------|----------------|--------------|---------------|------------|---------------------------|-----------------------------------|----|------|
| miR-146a-5p | 20510          | 128078       | 1252.38       | 6175.54    | 2.302                      | Up                                 | 0  | 0    |
| miR-342-3p  | 2117           | 7130         | 129.25        | 343.79     | 1.411                      | Up                                 | 0  | 0    |
| miR-29a-3p  | 42777          | 140623       | 2611.62       | 6780.42    | 1.376                      | Up                                 | 0  | 0    |
| miR-100-5p  | 132865         | 408192       | 8111.67       | 1968.79    | 1.278                      | Up                                 | 0  | 0    |
| miR-196a-5p | 473            | 8930         | 28.88         | 278.05     | 1.20E-303                  | Up                                 | 0  | 0    |
| miR-3529-5p | 1276           | 7983         | 77.9          | 248.57     | 1.673                      | Up                                 | 0  | 0    |
| miR-507c     | 11151          | 50968        | 680.79        | 1586.99    | 1.221                      | Up                                 | 0  | 0    |
| miR-196a-5p | 16653          | 101          | 1016.7        | 3.14       | -8.338                     | Down                               | 0  | 0    |

| miRNA id  | Count (control) | Count (IL6) | TPM (control) | TPM (IL6) | Log2 ratio (IL6/control) | Up/down-regulation (IL6/control) | P  | FDR  |
|---------|----------------|-------------|---------------|-----------|--------------------------|----------------------------------|----|------|
| miR-222-3p | 89564          | 48444       | 5468.06       | 2097.54   | -1.382                    | Down                              | 0  | 0    |
| miR-29a-3p  | 8451           | 5054        | 515.95        | 281.83    | -1.297                    | Down                              | 0  | 0    |
| miR-451a     | 2760           | 77297       | 168.5         | 3346.83   | 4.311                     | Up                                | 0  | 0    |

*P* values were corrected. TPM: Transcripts per kilobase million; FDR: False discovery rate; VCAM-1: Vascular cell adhesion molecule-1; TNF: Tumor necrosis factor; IL6: Interleukin 6.
### Table 3  Comparison of differentially expressed angiogenesis-related miRNAs between the TNFα group and control group

| miRNA id  | Count (control) | Count(TNFα) | TPM (control) | TPM (TNFα) | Log2 ratio (TNFα/control) | Up/ down-regulation (TNFα/control) | P         | FDR       |
|-----------|-----------------|-------------|---------------|-------------|---------------------------|-----------------------------------|-----------|-----------|
| hsa-miR-196a-5p | 26485          | 8359        | 1616.96       | 403.05      | -2.004253263              | Down                              | 0         | 0         |
| hsa-miR-320e   | 161            | 46          | 9.83          | 2.22        | -2.14663174               | Down                              | 5.95E-23  | 6.85E-22  |

*P*-values were corrected. TPM: Transcripts per kilobase million; FDR: False discovery rate; TNF: Tumor necrosis factor.

### Table 4  Comparison of differentially expressed angiogenesis-related miRNAs between the IL6 group and control group

| miRNA id   | Count (control) | Count (IL6) | TPM (control) | TPM (IL6) | Log2 ratio (IL6/control) | Up/down-regulation (IL6/control) | P         | FDR       |
|------------|-----------------|-------------|---------------|-----------|--------------------------|----------------------------------|-----------|-----------|
| hsa-miR-196a-5p | 26485          | 11440       | 1616.96       | 356.21    | -2.182484067              | Down                              | 0         | 0         |
| hsa-miR-17-5p | 252            | 138         | 15.39         | 4.3       | -1.839584667              | Down                              | 2.24E-35  | 2.98E-34  |
| hsa-miR-146b-5p | 78             | 44          | 4.76          | 1.37      | -1.79678568               | Down                              | 1.08E-11  | 7.53E-11  |
| hsa-miR-21-3p | 440            | 358         | 26.86         | 11.15     | -1.268415595              | Down                              | 4.66E-35  | 6.02E-34  |
| hsa-miR-320e | 161            | 153         | 9.83          | 4.76      | -1.046209843              | Down                              | 1.78E-10  | 1.11E-09  |

*P*-values were corrected. TPM: Transcripts per kilobase million; FDR: False discovery rate; IL6: Interleukin 6.

**Figure 4** Correlation analyses between groups. Blue color represents the correlation coefficient (the deeper the blue, the stronger the correlation). VCAM-1: Vascular cell adhesion molecule-1; TNF: Tumor necrosis factor; IL6: Interleukin 6.
Figure 5 Distribution of differentially expressed miRNAs in different groups. VCAM-1: Vascular cell adhesion molecule-1; TNF: Tumor necrosis factor; IL6: Interleukin 6.

Figure 6 Volcano plots. A-C: Volcano plots of (A) vascular cell adhesion molecule-1 group vs control group, (B) tumor necrosis factor α group vs control group, and (C) interleukin 6 group vs control group. The X axis is the log2(Fold change), and the Y axis is the -log10(FDR), with green points indicating down-regulation [log2(Fold change) ≤ -1 and FDR ≤ 0.001] and red points indicating upregulation [log2(Fold change) ≥ 1 and FDR ≤ 0.001]. FDR: False discovery rate.
Figure 7  Hierarchical clustering of differentially expressed miRNAs. A: Vascular cell adhesion molecule-1 group vs control group; B: Tumor necrosis factor α group vs control group; C: Interleukin 6 group vs control group. The X axis represents each pair of differences, and the Y axis represents differentially expressed miRNAs. The colors indicate the fold change, with red showing up-regulation, and blue showing down-regulation. TNF: Tumor necrosis factor; IL6: Interleukin 6.
### Table: Inflammatory factor-induced MSC exosome changes

| Category                        | Number of Genes |
|---------------------------------|-----------------|
| Biological adhesion             | 7140            |
| Cell junction                   | 530             |
| Membrane-enclosed lumen         | 410             |
| Extracellular region            | 8479            |
| Extracellular matrix            | 9963            |
| Extracellular matrix part       | 4952            |
| Extracellular region part       | 316             |
| Extracellular matrix part       | 316             |
| Nucleolus                       | 2822            |
| Membrane                        | 2469            |
| Membrane part                   | 5791            |
| Membrane-enclosed lumen         | 4313            |
| Membrane-enclosed lumen         | 8171            |
| Nuclear part                    | 8171            |
| Organelle                       | 8171            |
| Organelle part                  | 8171            |
| Synapse part                    | 8171            |
| Synapse                         | 8171            |
| Virion                           | 8171            |
| Virion part                     | 8171            |
| Antioxidant activity            | 8171            |
| Binding                         | 8171            |
| Catalytic activity              | 8171            |
| Channel regulator activity      | 8171            |
| Chemoattractant activity        | 8171            |
| Chemoattractant activity        | 8171            |
| Enzyme regulator activity       | 8171            |
| Guanyl-nucleotide exchange factor activity | 8171 |
| Metallochaperone activity       | 8171            |
| Molecular transducer activity   | 8171            |
| Nucleic acid binding transcription factor activity | 8171 |
| Nutrient reservoir activity     | 8171            |
| Protein binding transcription activity | 8171 |
| Protein tag                     | 8171            |
| Receptor activity               | 8171            |
| Receptor regulator activity     | 8171            |
| Structural molecule activity    | 8171            |
| Translation regulator activity  | 8171            |
| Transporter activity            | 8171            |
Figure 8  Gene ontology enrichment analysis of gene targets of differentially expressed miRNAs. A: Vascular cell adhesion molecule-1 group vs control group; B: Tumor necrosis factor α group vs control group; C: Interleukin 6 group vs control group. The X axis shows the number of differently expressed genes (their square root value), and the Y axis shows GO terms. All GO terms are grouped into three ontologies: Blue indicates biological process, brown indicates cellular components, and red indicates molecular function.
Inflammatory factor-induced MSC exosome changes

> Cell growth and death
> Cell motility
> Cellular community-eukaryotes
> Transport and catabolism
> Membrane transport
> Signaling molecules and interaction
> Signal transduction
> Folding, sorting and degradation
> Replication and repair
> Transcription
> Translation
> Cancers: Overview
> Cancers: Specific types
> Cardiovascular diseases
> Drug resistance: Antineoplastic
> Endocrine and metabolic diseases
> Immune diseases
> Infectious diseases: Bacterial
> Infectious diseases: Parasitic
> Infectious diseases: Viral
> Neurodegenerative diseases
> Substance dependence
> Amino acid metabolism
> Biosynthesis of other secondary metabolites
> Carbohydrate metabolism
> Energy metabolism
> Glioblastoma and glioma
> Glycan biosynthesis and metabolism
> Lipid metabolism
> Metabolism of cofactors and vitamins
> Metabolism of other amino acids
> Metabolism of terpenoids and polyketides
> Nucleotide metabolism
> Xenobiotics biodegradation and metabolism
> Aging
> Circulatory system
> Development
> Digestive system
> Endocrine system
> Environmental adaptation
> Excretory system
> Immune system
> Nervous system
> Sensory system

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Figure 9  Pathway enrichment analysis of gene targets of differentially expressed miRNAs. A: Comparison of the top 20 enriched pathway terms between vascular cell adhesion molecule-1 (VCAM-1) group and control group; B: Comparison of the Kyoto Encyclopedia of Genes and Genomes (KEGG) classification between VCAM-1 group and control group; C: Comparison of the top 20 enriched pathway terms between tumor necrosis factor (TNF) α group and control group; D: Comparison of the KEGG classification between TNFα group and control group; E: Comparison of the top 20 enriched pathway terms between interleukin 6 (IL6) group and control group; F: Comparison of the KEGG classification between IL6 group and control group. A, C, and E: The top 20 enriched pathway terms displayed as scatterplots. The rich factor is the ratio of target gene numbers annotated in this pathway term to all gene numbers annotated in this pathway term. The greater the rich factor, the greater the degree of enrichment. The Q-value is the corrected P-value and ranges from 0-1; the lower the Q-value, the greater the level of enrichment; B, D, and F: The X axis shows the number of target genes, and the Y axis shows the second KEGG pathway terms. The first pathway terms are indicated using different colors. The second pathway terms are subgroups of the first pathway terms and are grouped together on the X axis on the right side.
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**A**
Gene ontology enrichment analysis of the relative angiogenesis gene (VCAM1 Data)

**B**
Gene ontology enrichment analysis of the relative angiogenesis gene (TNFα Data)

**C**
Gene ontology enrichment analysis of the relative angiogenesis gene (TNFα Data)
Figure 10  Gene ontology enrichment analysis of angiogenesis-related genes. A: The number of angiogenesis-related genes in the vascular cell adhesion molecule-1 (VCAM-1) group; B: The proportion of angiogenesis-related genes in the VCAM-1 group; C: The number of angiogenesis-related genes in the tumor necrosis factor (TNF) α group; D: The proportion of angiogenesis-related genes in the TNFα group; E: The number of angiogenesis gene distributions in the interleukin 6 (IL6) group; F: The proportion of angiogenesis-related genes in the IL6 group; G: Comparison of the number of angiogenesis-related genes in the three groups to that of the control group.
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Figure 11 Regulatory mechanism of the PI3K-AKT signal pathway in different groups. A: Vascular cell adhesion molecule-1 group; B: Tumor necrosis factor α group; C: Interleukin 6 group. Up-regulated genes are marked with red borders and down-regulated genes with green borders. Unchanged genes are marked with black borders.
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Figure 12 Regulatory mechanism of the MAPK signal pathway in different groups. A: Vascular cell adhesion molecule-1 group; B: Tumor necrosis factor α group; C: Interleukin 6 group. Up-regulated genes are marked with red borders and down-regulated genes with green borders. Unchanged genes are marked with black borders.
Figure 13 Regulatory mechanism of the VEGF signal pathway in different groups. A: Vascular cell adhesion molecule-1 group; B: Tumor necrosis factor α group; C: Interleukin 6 group. Up-regulated genes are marked with red borders and down-regulated genes with green borders. Unchanged genes are marked with black borders.
Figure 14  Tube formation in endothelial cells treated with mesenchymal stem cell exosomes. A: Micrograph showing human umbilical vein endothelial cells (HUVECs) cultured on Matrigel-coated plates in medium with phosphate buffered saline (control); B: Micrograph showing HUVECs cultured on Matrigel-coated plates in medium with mesenchymal stem cell exosomes (MSC-exo); C: Micrograph showing HUVECs cultured on Matrigel-coated plates in medium with MSCs-exo stimulated with tumor necrosis factor α; D: Micrograph showing HUVECs cultured on Matrigel-coated plates in medium with MSCs-exo stimulated with interleukin 6; E: The number of tubes formed in each group; F: The number of branching points in each group.
Western blot analysis.

Western blot was performed to detect the expression of the indicated proteins in endothelial cells treated with phosphate buffered saline (control), mesenchymal stem cell exosomes (MSCs-exo), MSCs-exo TNFα (stimulated with tumor necrosis factor α), MSCs-exo IL6 (stimulated with interleukin 6). GAPDH was used as an internal loading control. TNF: Tumor necrosis factor; IL6: Interleukin 6.

ARTICLE HIGHLIGHTS

Research background
Stem cell transplantation has been developing rapidly and has resulted in breakthroughs for the treatment of various diseases.

Research motivation
Treatments utilizing stems cells often require stem cells to be exposed to inflammatory environments, such as vascular cell adhesion molecule-1, tumor necrosis factor α (TNFα), and interleukin 6 (IL6). Stem cell-derived exosomes are especially important in producing miRNAs that impact angiogenesis.

Research objectives
MicroRNAs (miRNAs) are RNAs 0-20 nucleotides in length, which are derived from hairpin-like precursor miRNAs. They acts as important regulators of mRNA expression. It has been reported that miRNAs play critical roles in some cells and have the potential as diagnostic and therapeutic biomarkers.

Research methods
The morphology and quantity of mesenchymal stem cell (MSC) exosomes (MSCs-exo) are influenced by different inflammatory cytokine environments.

Research results
The morphology and quantity of each group of MSC exosomes were observed and measured. The miRNAs in MSCs-exo were sequenced. Differential expression of miRNAs and their target genes as well as the related regulatory mechanisms were researched.

Research conclusions
TNFα and IL6 may influence the expression of miRNAs that down-regulate the PI3K-AKT, MAPK, and VEGF signaling pathways; particularly, IL6 significantly down-regulates the PI3K-AKT signaling pathway.

Research perspectives
Overall, inflammatory cytokines may lead to changes in exosomal miRNAs that abnormally impact cellular components, molecular function, and biological process, particularly angiogenesis.

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