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ORIGINAL ARTICLE

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),

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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

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[6]. MiRNAs are non-coding RNAs 22-25 nucleotides in length[7-10]. Exosomes are especially important in producing miRNAs that impact angiogenesis[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 (1.0×10^5 cells/mL) were cultured in a serum-free DMEM/F12 (Sigma-Aldrich, United States) medium for 48 h; VCAM-1 group: MSCs (1.0×10^5 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 (1.0×10^5 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 (1.0×10^5 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[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 precipitate was next rinsed with phosphate buffered saline (PBS) and ultracentrifuged at 100000 × *g* for 70 min at 4 °C. 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 pheatmap. 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 (4.0×10^4, serum-starved overnight) were seeded in a 96-well plate, cultured in 5% CO_2 overnight, and then treated with PBS (control), control scrambled MSCs-exo, MSCs-exo$^{TNF\alpha}$ (5×10^9, stimulated with TNFα), or MSCs-exo^{IL6} (5×10^9, stimulated with IL6). The plates were previously coated with 150 μL of growth factor-reduced Matrigel (356234, Corning, United States) in serum-free medium. Tube formation ability of control or MSCs-exo-treated HUVECs was examined by determining the total number of tubes formed and branching points in 4 to 6 h. Each condition in each experiment was assessed at least in duplicate.

Western blot analysis

HUVECs (4.0×10^4) were treated with PBS (control), control scrambled MSCs-exo, MSCs-exo$^{TNF\alpha}$ (5×10^9, stimulated with TNFα), or MSCs-exo^{IL6} (5×10^9, stimulated with IL6). The effect of MSCs-exo treatment on PI3K-AKT and MAPK, which are related to angiogenic signaling, was examined by measuring the expression of AKT (1:1000 dilution, 4691S, Cell Signaling Technology, Danvers, MA, United States), phospho-AKT (1:1000 dilution, 13038S, Cell Signaling Technology), phospho-p44/42 MAPK (Erk1/2) (1:2000 dilution, 4370S, Cell Signaling Technology), and p44/42 MAPK (Erk1/2) (1:1000 dilution, 4695S, Cell Signaling Technology) in endothelial cells by Western blot. Each condition in each experiment was assessed at least in triplicate.

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 7.42×10^8/mL in the control group, 1.10×10^9/mL in the VCAM-1 group, 7.37×10^8/mL in the TNFα group, and 3.01×10^8/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

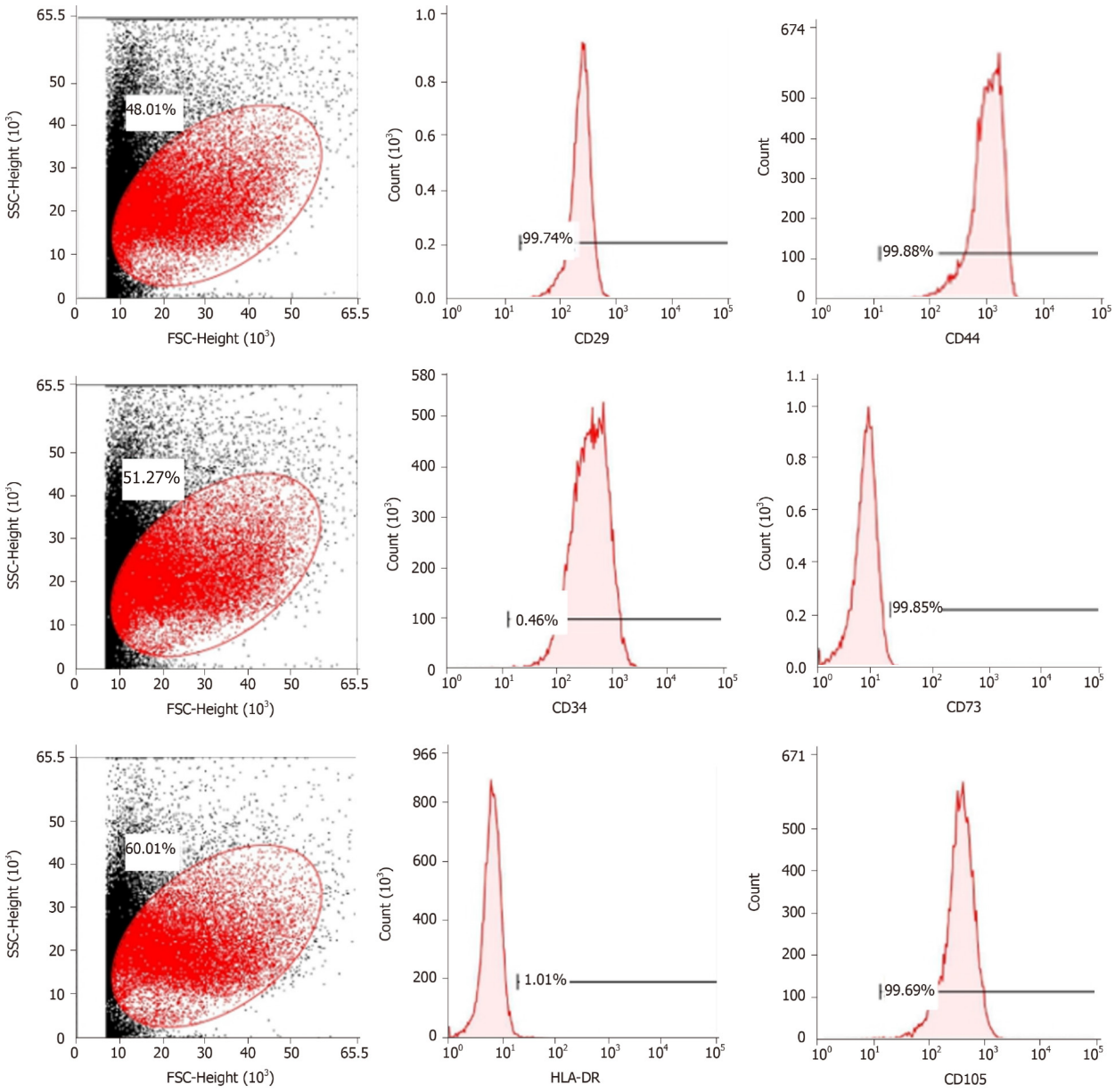


Figure 1 Flow cytometric analysis of mesenchymal stem cell-related cell surface markers. High expression of positive mesenchymal cell markers (CD29, CD44, CD73, and CD105), and low expression of negative cell markers such as CD34 and HLA-DR were observed. n = 3 to 5; P < 0.05,

(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 down-regulated 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).

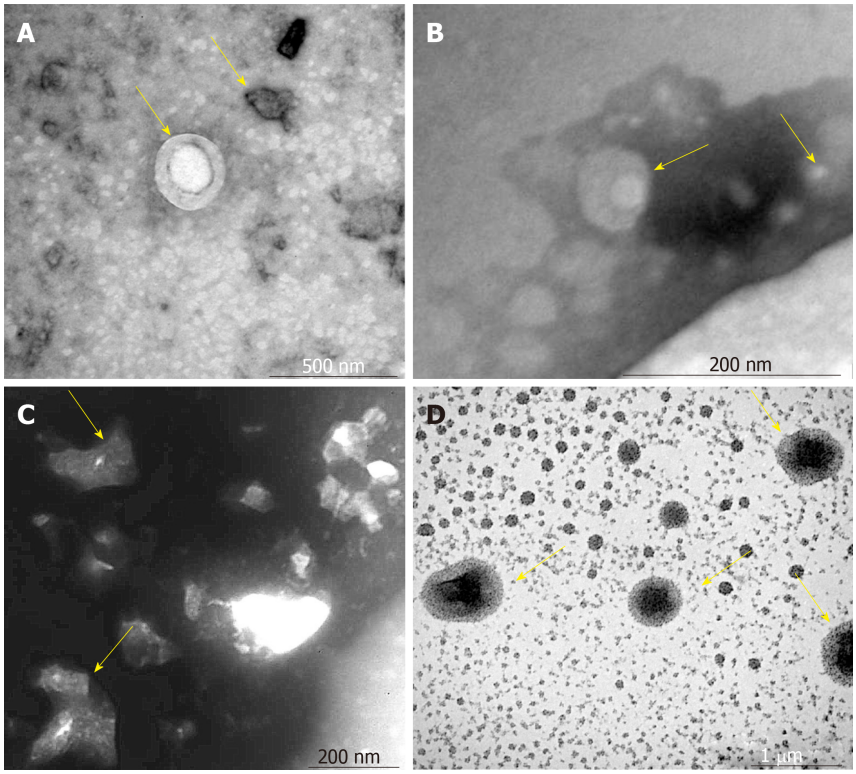


Figure 2 Morphology of mesenchymal stem cell exosomes (magnification, ×65000). A-D: Mesenchymal stem cell exosomes (MSCs-exo) in (A) the control group (yellow arrow), (B) the vascular cell adhesion molecule-1 group (yellow arrow), (C) the tumor necrosis factor α group (yellow arrow), and (D) the interleukin 6 group (yellow arrow).

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

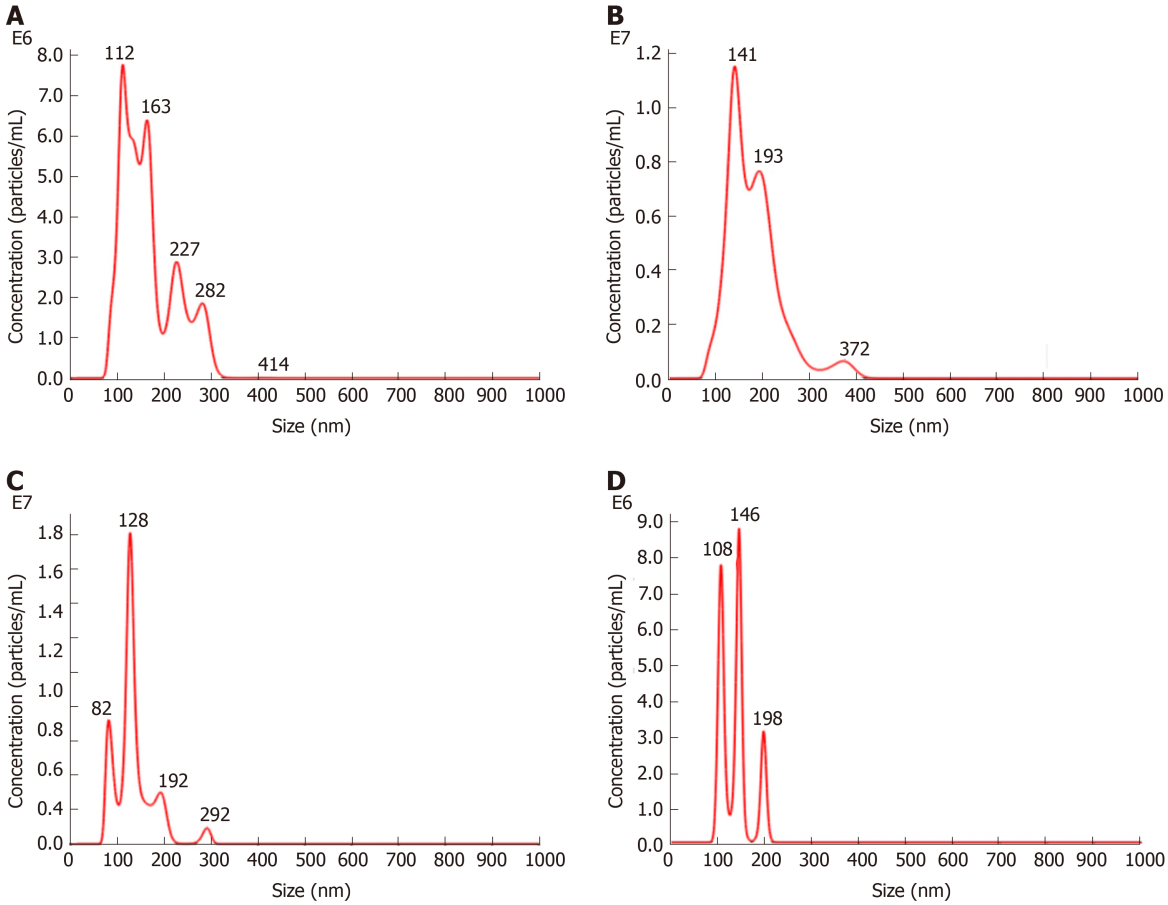


Figure 3 Nanoparticle tracking analysis. A-D: Density of exosomes of different sizes in (A) the control group, (B) the vascular cell adhesion molecule-1 group, (C) the tumor necrosis factor α group, and (D) the interleukin 6 group.

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

Top 10 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	sRNA id	Count (32116232)
miR-21-5p	1544110	76933.99	miR-21-5p	1640334	70885.38	miR-21-5p	1454726	70066.87	miR-21-5p	1323387
miR-127-3p	159283	9724.54	miR-127-3p	259830	11250.19	miR-100-5p	408192	19681.79	miR-199a-3p,miR-199b-3p	317377
miR-199a-3p,miR-199b-3p	139220	8499.65	miR-199a-3p, miR-199b-3p	155376	6727.51	miR-127-3p	321306	15492.41	miR-127-3p	300549
miR-100-5p	132865	8111.67	miR-100-5p	140774	6095.27	miR-199a-3p, miR-199b-3p	271902	13110.3	miR-100-5p	265054
miR-222-3p	89564	5468.06	miR-26a-5p	107800	4667.55	miR-23a-3p	140623	6780.42	miR-34a-5p	178542
miR-26a-5p	77524	4732.99	miR-34a-5p	86786	3757.68	miR-222-3p	129932	6264.93	miR-23a-3p	144385
miR-34a-5p	76681	4681.52	miR-451a	77297	3346.83	miR-146a-5p	128078	6175.54	miR-222-3p	111957
miR-181a-5p	67287	4108	miR-23a-3p	71930	3114.44	miR-34a-5p	119952	5783.73	miR-146a-5p	100038
miR-411-5p	56606	3455.91	miR-196a-5p	63939	2768.45	miR-143-3p	101398	4889.11	miR-26a-5p	98114
miR-143-3p	49739	3036.66	miR-181a-5p	62752	2717.05	miR-26a-5p	99628	4803.76	miR-181a-5p	97685

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)

Comparison of top 10 differentially expressed miRNAs																										
VCAM-1 group *vs* control group									TNFα group *vs* control group								IL6 group *vs* control group									
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	miRNA id	Count (control)	Count (TNFα)	TPM (control)	TPM (TNFα)	Log2 ratio (TNFα/control)	Up/down-regulation (TNFα/control)	*P*	FDR	miRNA id	Count (control)	Count (IL6)	TPM (control)	TPM (IL6)	Log2 ratio (IL6/control)	Up/down-regulation (IL6/control)	*P* value	FDR
miR-146a-5p	20510	5173	1252.18	223.98	-2.483	Down	0	0	miR-146a-5p	20510	128078	1252.18	6175.54	2.302123	Up	0	0	miR-3529-3p	473	8930	28.88	278.05	3.267202	Up	0	0
miR-149-5p	4558	2391	278.27	103.53	-1.42644	Down	0	0	miR-342-3p	2117	7130	129.25	343.79	1.411363	Up	0	0	miR-320c	1276	7983	77.9	248.57	1.673957	Up	0	0
miR-222-3p	89564	48444	5468.06	2097.54	-1.38233	Down	0	0	miR-23a-3p	42777	140623	2611.62	6780.42	1.37643	Up	0	0	miR-24-3p	11151	50968	680.79	1586.99	1.221011	Up	0	0
miR-29a-3p	8451	5054	515.95	218.83	-1.23742	Down	0	0	miR-100-5p	132865	408192	8111.67	19681.79	1.278791	Up	0	0	miR-7-5p	16653	101	1016.7	3.14	-8.33891	Down	0	0
miR-451a	2760	77297	168.5	3346.83	4.311975	Up	0	0	miR-337-3p	5655	16027	345.25	772.77	1.162398	Up	0	0	miR-196a-5p	26485	11440	1616.96	356.21	-2.18248	Down	0	0
miR-490-5p	1839	475	112.27	20.57	-2.44836	Down	2.00E-305	1.20E-303	miR-409-3p	4207	11169	256.85	538.54	1.068127	Up	0	0	miR-451a	2760	1658	168.5	51.62	-1.70675	Down	0	0
miR-1246	1812	6191	110.63	268.06	1.276813	Up	1.39E-276	8.02E-275	miR-221-3p	4067	10541	248.3	508.26	1.033482	Up	0	0	miR-379-5p	13430	8083	819.93	251.68	-1.70391	Down	0	0
miR-181a-2-3p	3400	2008	207.58	86.94	-1.25558	Down	2.31E-219	1.18E-217	miR-7-5p	16653	136	1016.7	6.56	-7.27598	Down	0	0	miR-196b-5p	18735	15676	1143.81	488.1	-1.2286	Down	0	0
miR-204-5p	3116	1757	190.24	76.08	-1.32223	Down	3.36E-218	1.66E-216	miR-196a-5p	26485	8359	1616.96	403.05	-2.00425	Down	0	0	miR-424-5p	12774	11917	779.88	371.06	-1.0716	Down	0	0
miR-378e	2	817	0.12	35.37	8.203348	Up	4.36E-186	2.00E-184	miR-26b-5p	6496	3410	396.59	164.42	-1.27026	Down	0	0	miR-26b-5p	6496	6086	396.59	189.5	-1.06545	Down	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	-1.046229843	Down	1.78E-10	1.11E-09

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

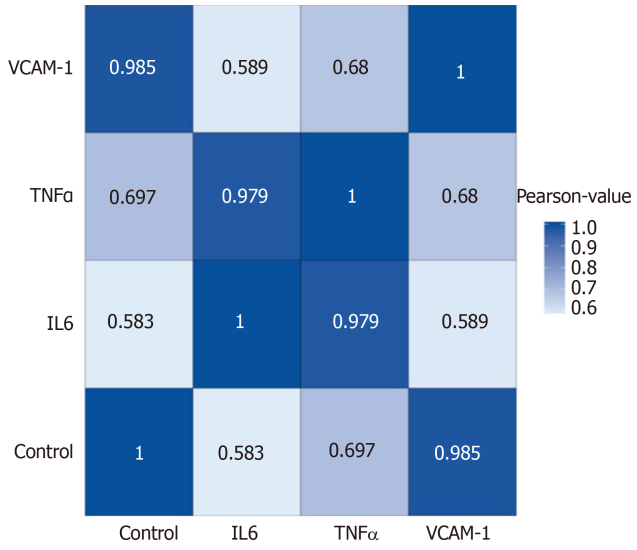


Figure 4 Correlation analyses between groups. Blue color represents the correlation coefficient (the deeper the blue, the stronger the correlation). VCAM-1: Vascular cell adhesion molecule-1; TNF: Tumor necrosis factor; IL6: Interleukin 6.

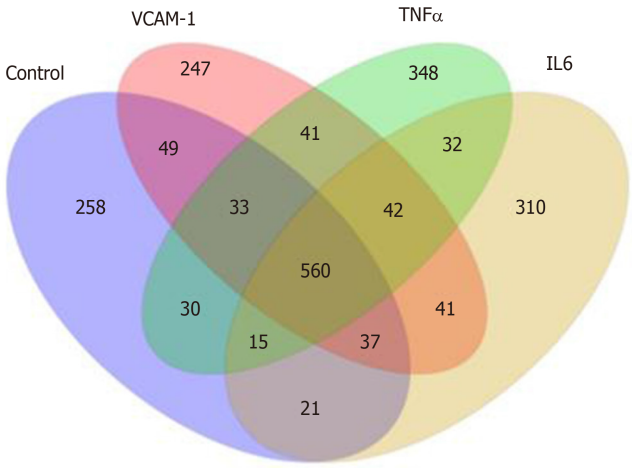


Figure 5 Distribution of differentially expressed miRNAs in different groups. VCAM-1: Vascular cell adhesion molecule-1; TNF: Tumor necrosis factor; IL6: Interleukin 6.

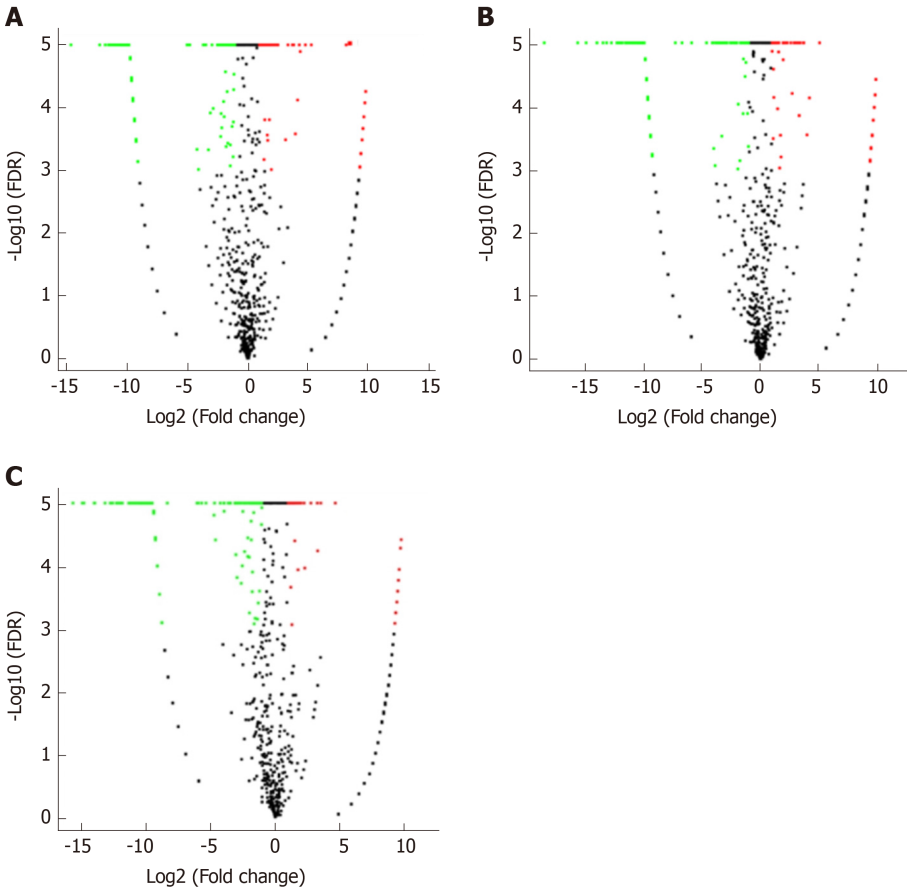


Figure 6 Volcano plots. A-C: Volcano plots of (A) vascular cell adhesion molecule-1 group *vs* control group, (B) tumor necrosis factor α group *vs* control group, and (C) interleukin 6 group *vs* control group. The X axis is the log2(Fold change), and the Y axis is the -log10(FDR), with green points indicating down-regulation [log2(Fold old change) ≤ -1 and FDR ≤ 0.001] and red points indicating upregulation [log2(Fold change) ≥ 1 and FDR ≤ 0.001]. FDR: False discovery rate.

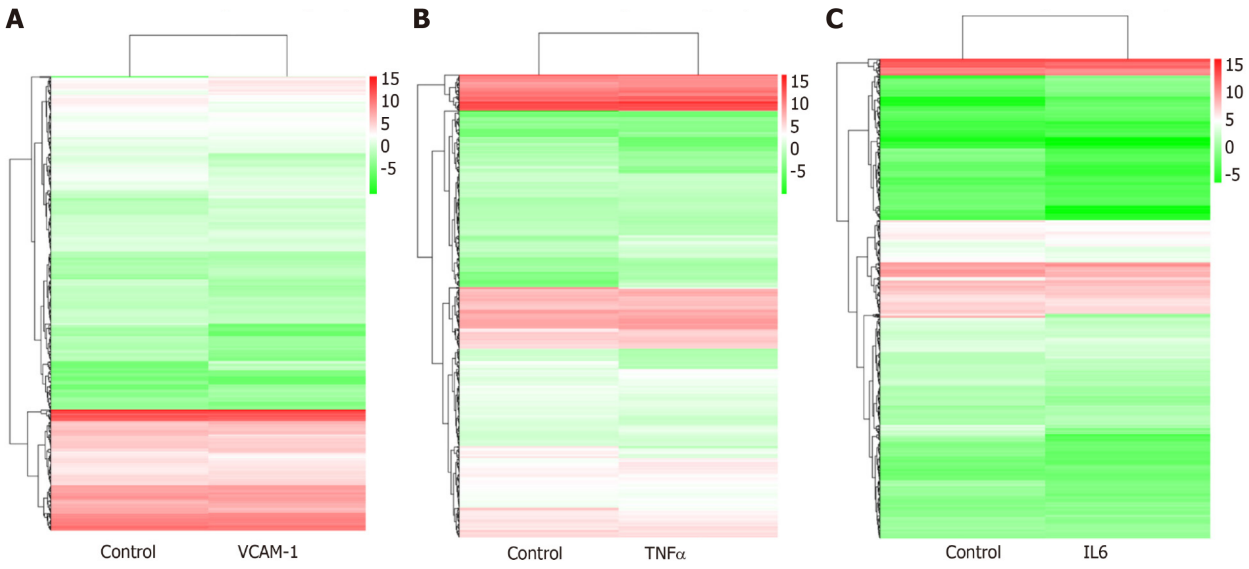
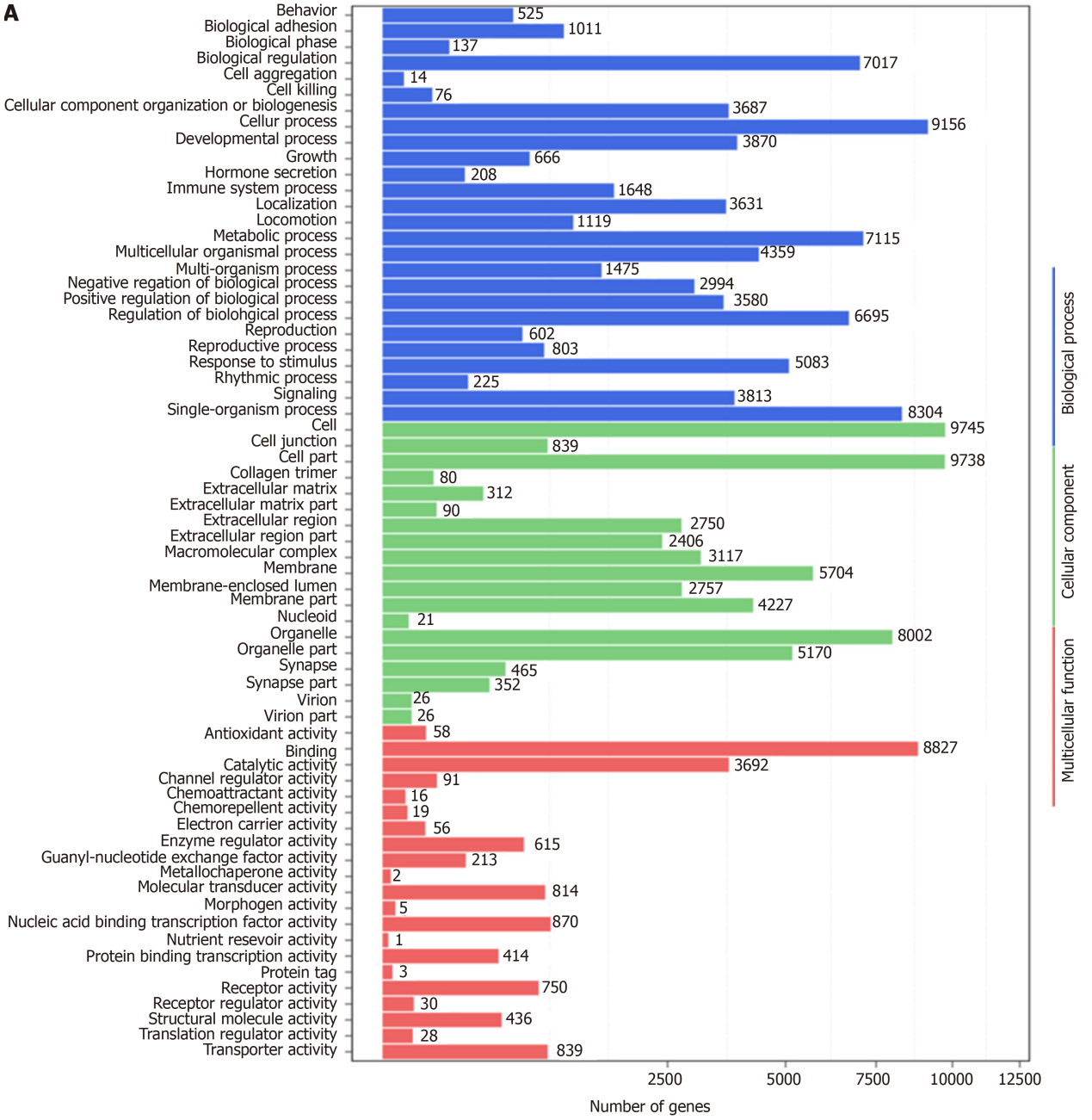


Figure 7 Hierarchical clustering of differentially expressed miRNAs. A: Vascular cell adhesion molecule-1 group *vs* control group; B: Tumor necrosis factor α group *vs* control group; C: Interleukin 6 group *vs* control group. The X axis represents each pair of differences, and the Y axis represents 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.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Behavior 525
Biological adhesion 1011
Biological phase 137
Biological regulation 7017
Cell aggregation 14
Cell killing 76
Cellular component organization or biologenesis 3687
Cellur process 9156
Developmental process 3870
Growth 666
Hormone secretion 208
Immune system process 1648
Localization 3631
Locomotion 1119
Metabolic process 7115
Multicellular organismal process 4359
Multi-organism process 1475
Negative regation of biological process 2994
Positive regulation of biological process 3580
Regulation of biolohgical process 6695
Reproduction 602
Reproductive process 803
Response to stimulus 5083
Rhythmic process 225
Signaling 3813
Single-organism process 8304
Cell 9745
Cell junction 839
Cell part 9738
Collagen trimer 80
Extracellular matrix 312
Extracellular matrix part 90
Extracellular region 2750
Extracellular region part 2406
Macromolecular complex 3117
Membrane 5704
Membrane-enclosed lumen 2757
Membrane part 4227
Nucleoid 21
Organelle 8002
Organelle part 5170
Synapse 465
Synapse part 352
Virion 26
Virion part 26
Antioxidant activity 58
Binding 8827
Catalytic activity 3692
Channel regulator activity 91
Chemoattractant activity 16
Chemorepellent activity 19
Electron carrier activity 56
Enzyme regulator activity 615
Guanyl-nucleotide exchange factor activity 213
Metallochaperone activity 2
Molecular transducer activity 814
Morphogen activity 5
Nucleic acid binding transcription factor activity 870
Nutrient resevoir activity 1
Protein binding transcription activity 414
Protein tag 3
Receptor activity 750
Receptor regulator activity 30
Structural molecule activity 436
Translation regulator activity 28
Transporter activity 839
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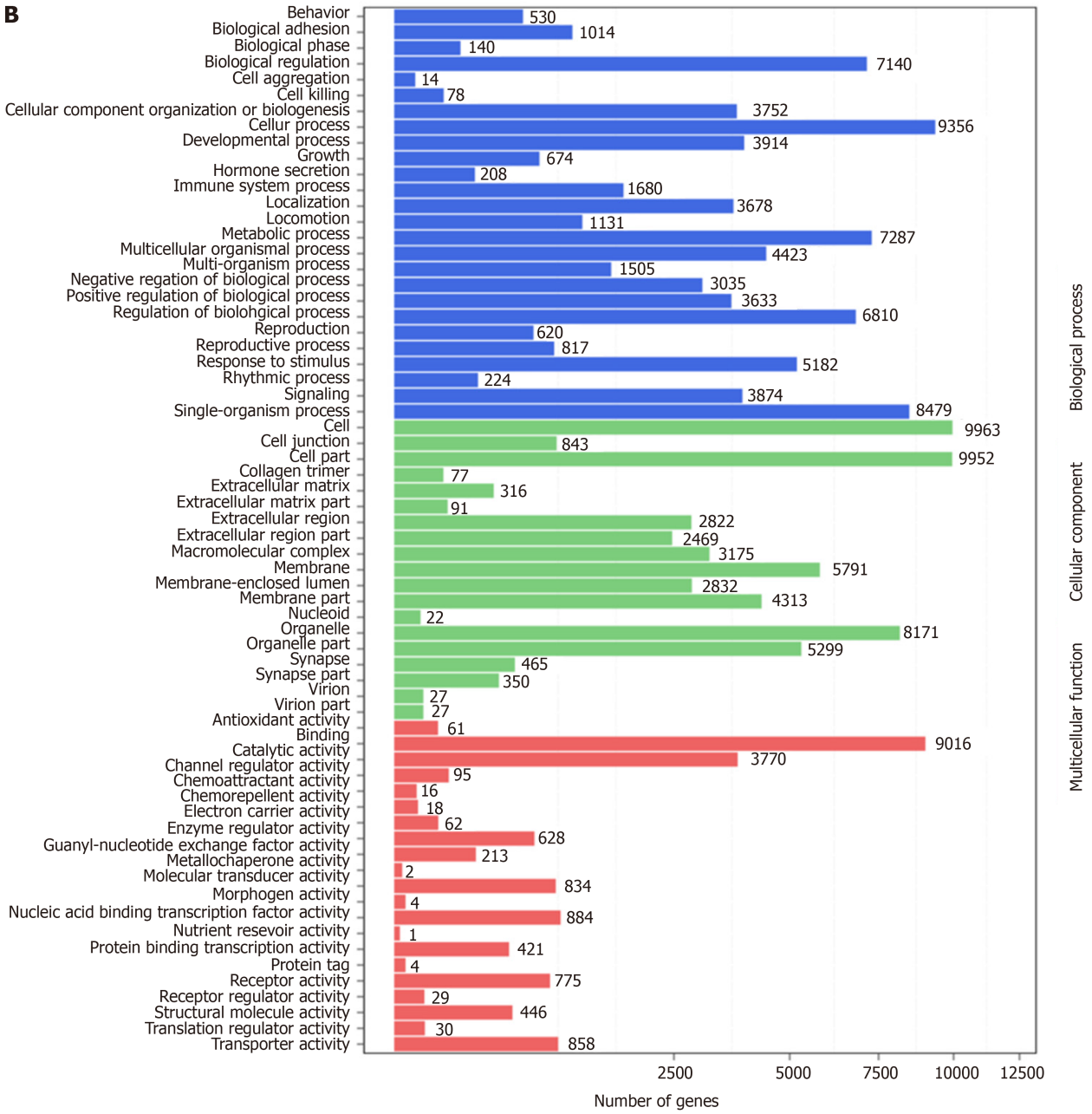
5000
7500
10000
12500
Number of genes
Biological process
Cellular component
Multicellular function


B
Behavior 530
Biological adhesion 1014
Biological phase 140
Biological regulation 7140
Cell aggregation 14
Cell killing 78
Cellular component organization or biologenesis 3752
Cellur process 9356
Developmental process 3914
Growth 674
Hormone secretion 208
Immune system process 1680
Localization 3678
Locomotion 1131
Metabolic process 7287
Multicellular organismal process 4423
Multi-organism process 1505
Negative regation of biological process 3035
Positive regulation of biological process 3633
Regulation of biolohgical process 6810
Reproduction 620
Reproductive process 817
Response to stimulus 5182
Rhythmic process 224
Signaling 3874
Single-organism process 8479
Cell 9963
Cell junction 843
Cell part 9952
Collagen trimer 77
Extracellular matrix 316
Extracellular matrix part 91
Extracellular region 2822
Extracellular region part 2469
Macromolecular complex 3175
Membrane 5791
Membrane-enclosed lumen 2832
Membrane part 4313
Nucleoid 22
Organelle 8171
Organelle part 5299
Synapse 465
Synapse part 350
Virion 27
Virion part 27
Antioxidant activity 61
Binding 9016
Catalytic activity 3770
Channel regulator activity 95
Chemoattractant activity 16
Chemorepellent activity 18
Electron carrier activity 62
Enzyme regulator activity 628
Guanyl-nucleotide exchange factor activity 213
Metallochaperone activity 2
Molecular transducer activity 834
Morphogen activity 4
Nucleic acid binding transcription factor activity 884
Nutrient resevoir activity 1
Protein binding transcription activity 421
Protein tag 4
Receptor activity 775
Receptor regulator activity 29
Structural molecule activity 446
Translation regulator activity 30
Transporter activity 858
2500
5000
7500
10000
12500
Number of genes
Biological process
Cellular component
Multicellular function

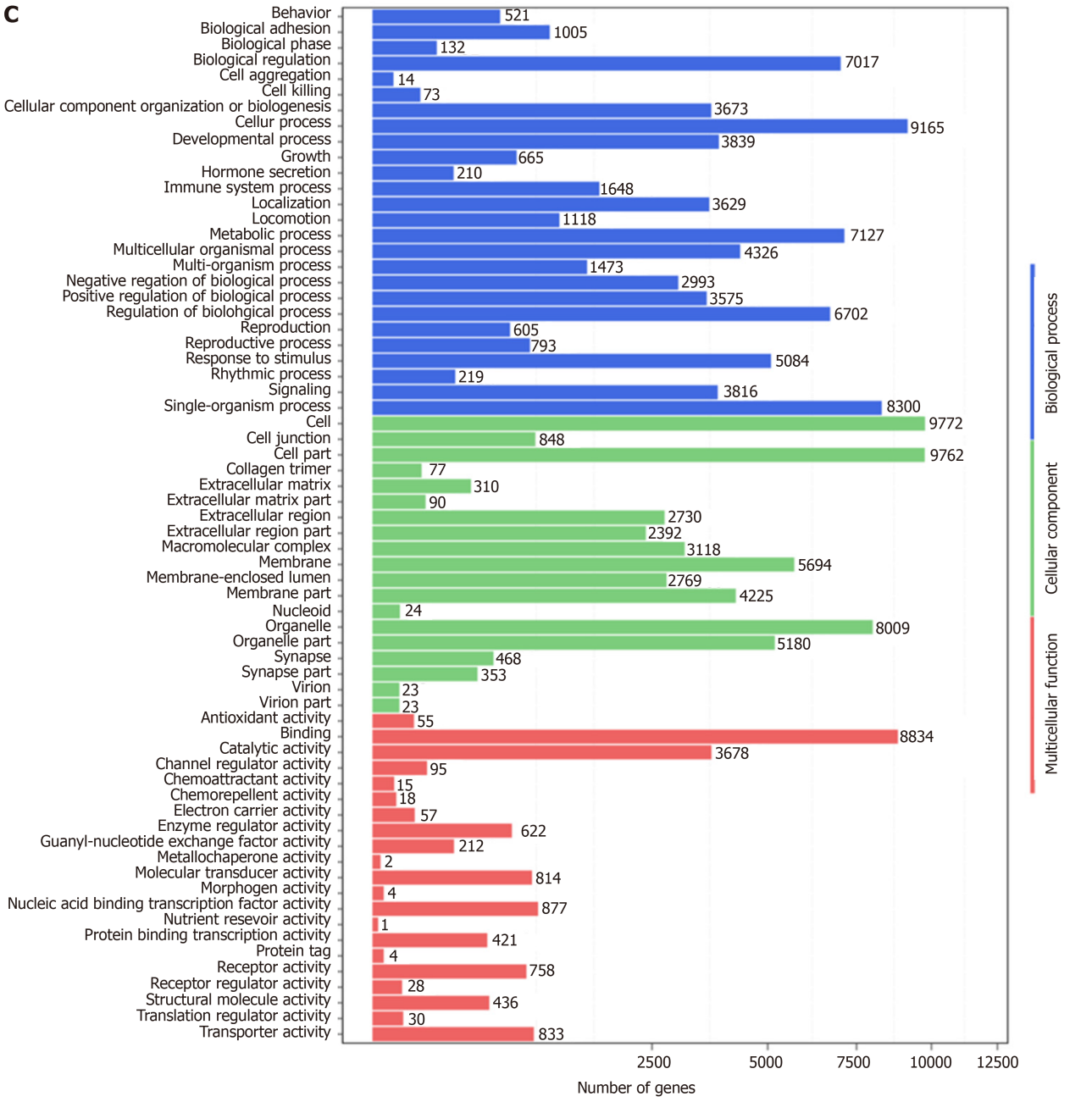
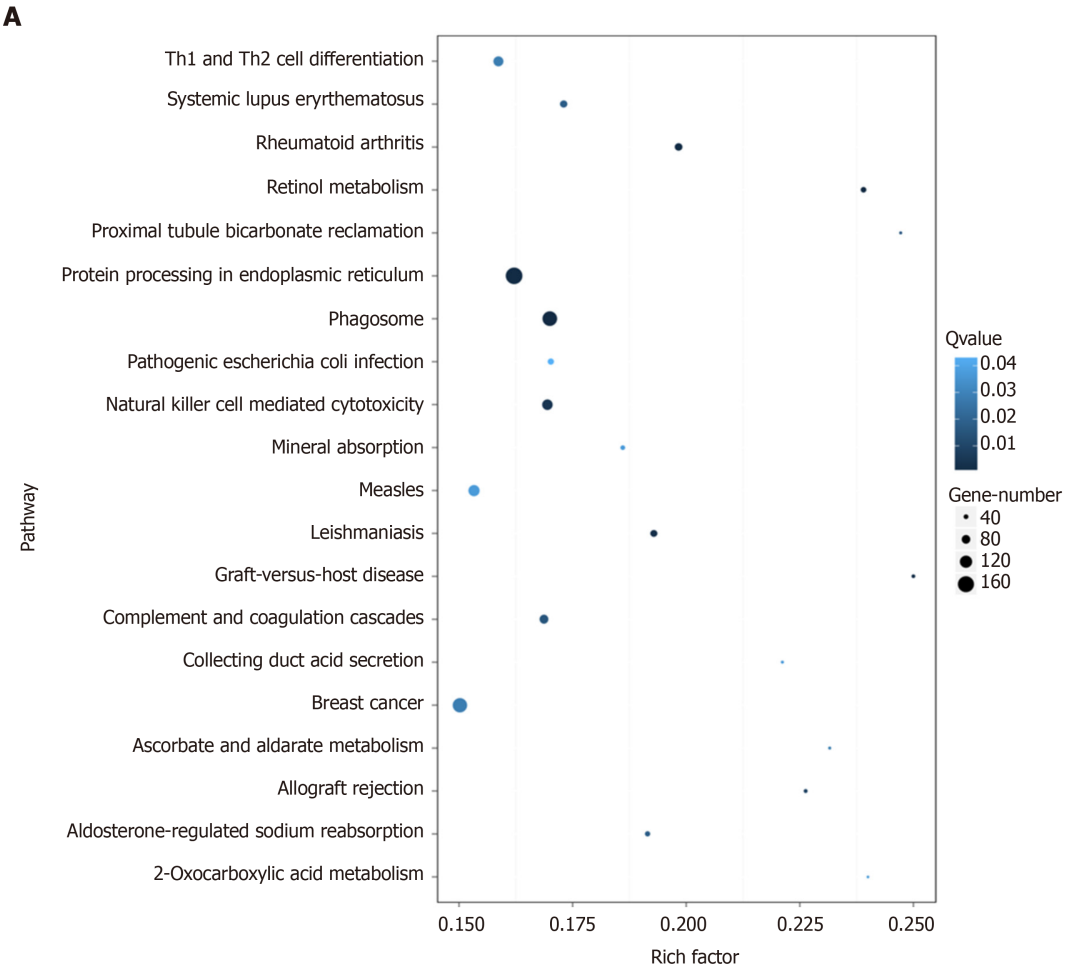


Figure 8 Gene ontology enrichment analysis of gene targets of differentially expressed miRNAs. A: Vascular cell adhesion molecule-1 group *vs* control group; B: Tumor necrosis factor α group *vs* control group; C: Interleukin 6 group *vs* control group. The X axis shows the number of differently expressed 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

A


Th1 and Th2 cell differentiation
Systemic lupus eryrthematosus
Rheumatoid arthritis
Retinol metabolism
Proximal tubule bicarbonate reclamation
Protein processing in endoplasmic reticulum
Phagosome
Pathogenic escherichia coli infection
Natural killer cell mediated cytotoxicity
Mineral absorption
Measles
Leishmaniasis
Graft-versus-host disease
Complement and coagulation cascades
Collecting duct acid secretion
Breast cancer
Ascorbate and aldarate metabolism
Allograft rejection
Aldosterone-regulated sodium reabsorption
2-Oxocarboxylic acid metabolism
Pathway
0.150
0.175
0.200
0.225
0.250
Rich factor
Qvalue
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0.03
0.02
0.01
Gene-number
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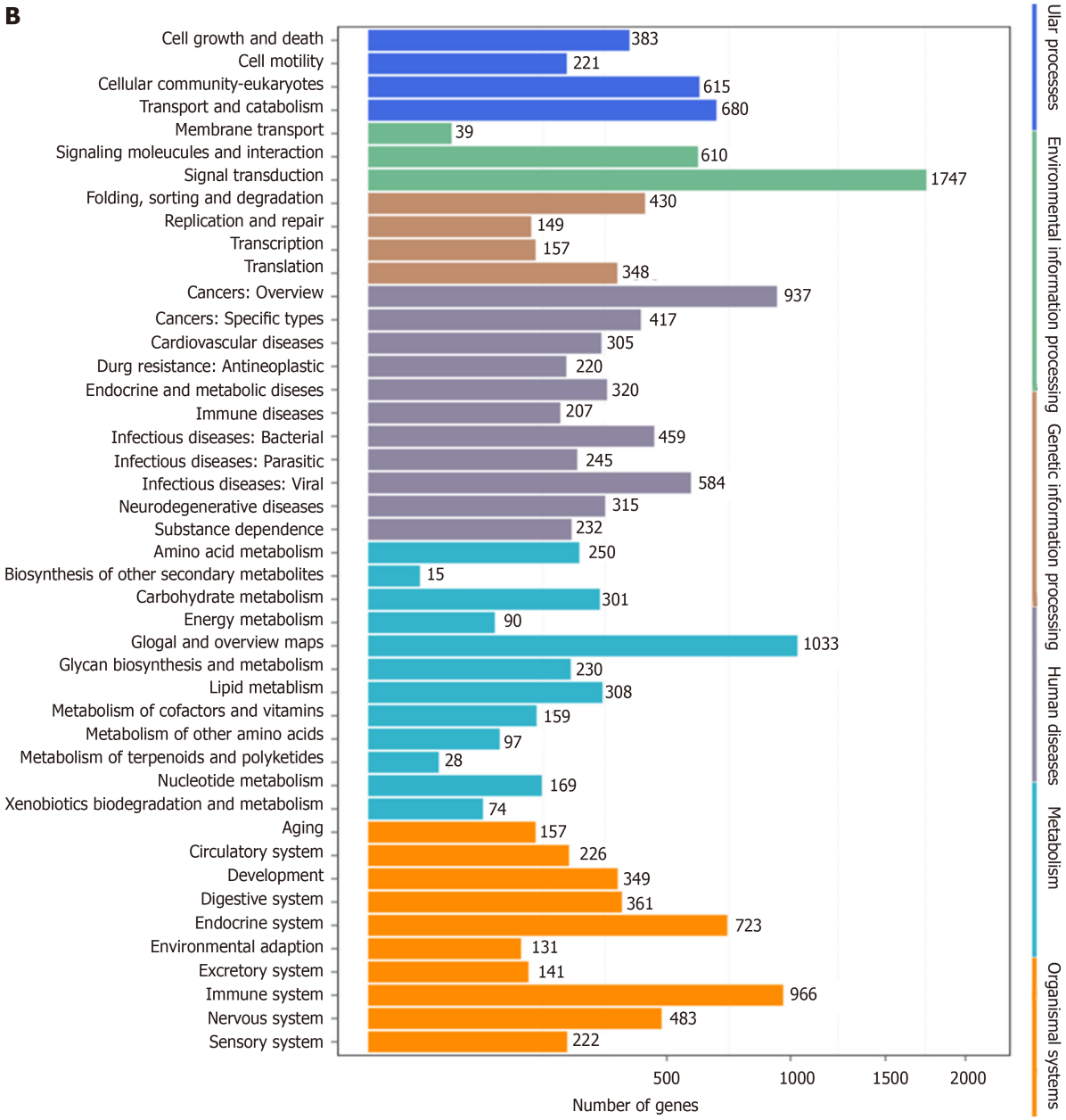
80
120
160


B
Cell growth and death 383
Cell motility 221
Cellular community-eukaryotes 615
Transport and catabolism 680
Membrane transport 39
Signaling moleucules and interaction 610
Signal transduction 1747
Folding, sorting and degradation 430
Replication and repair 149
Transcription 157
Translation 348
Cancers: Overview 937
Cancers: Specific types 417
Cardiovascular diseases 305
Durg resistance: Antineoplastic 220
Endocrine and metabolic diseses 320
Immune diseases 207
Infectious diseases: Bacterial 459
Infectious diseases: Parasitic 245
Infectious diseases: Viral 584
Neurodegenerative diseases 315
Substance dependence 232
Amino acid metabolism 250
Biosynthesis of other secondary metabolites 15
Carbohydrate metabolism 301
Energy metabolism 90
Glogal and overview maps 1033
Glycan biosynthesis and metabolism 230
Lipid metablism 308
Metabolism of cofactors and vitamins 159
Metabolism of other amino acids 97
Metabolism of terpenoids and polyketides 28
Nucleotide metabolism 169
Xenobiotics biodegradation and metabolism 74
Aging 157
Circulatory system 226
Development 349
Digestive system 361
Endocrine system 723
Environmental adaption 131
Excretory system 141
Immune system 966
Nervous system 483
Sensory system 222
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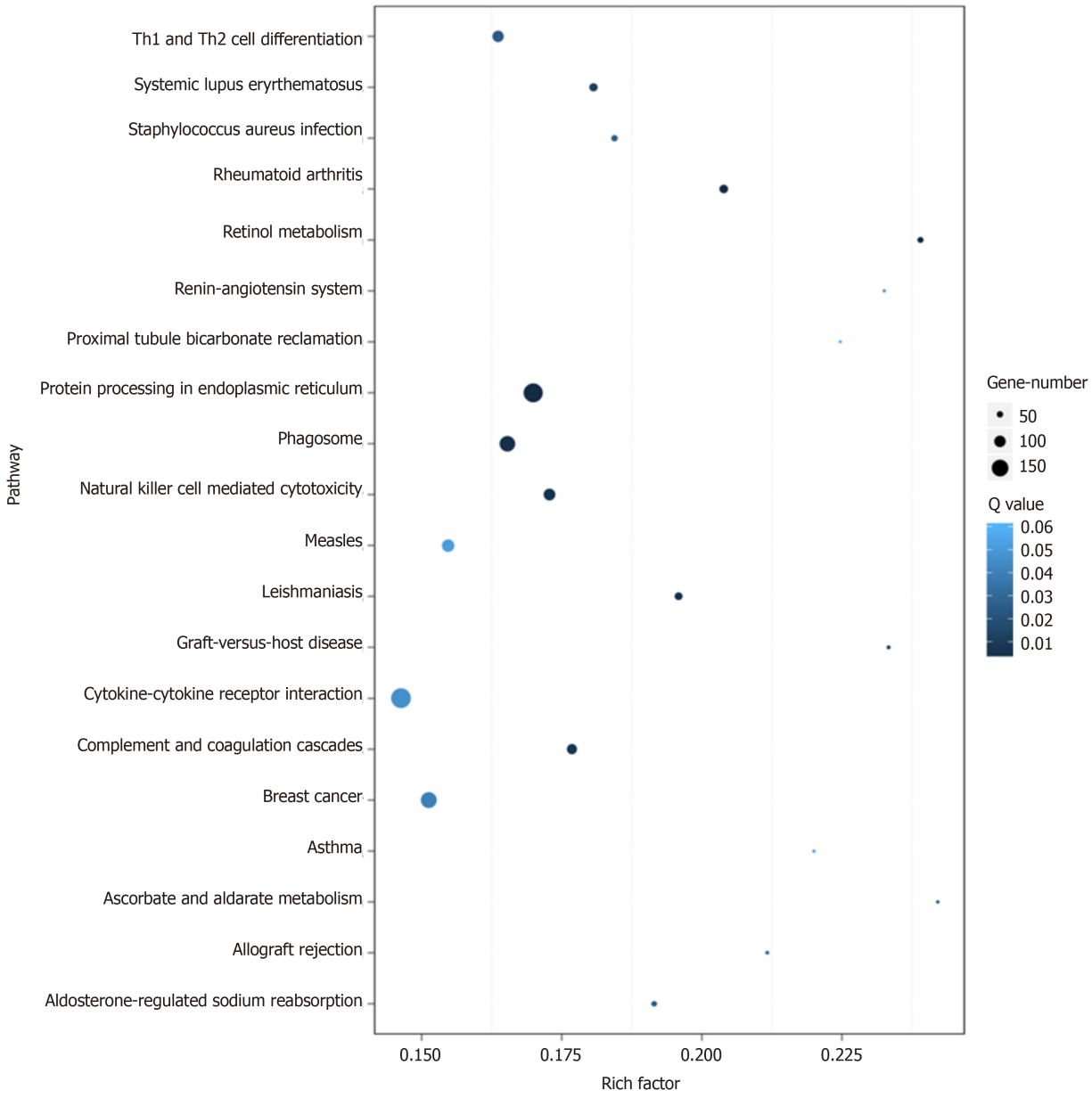
1000
1500
2000
Number of genes
Ular processes
Environmental information processing
Genetic information processing
Human diseases
Metabolism
Organismal systems

C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Systemic lupus eryrthematosus
Staphylococcus aureus infection
Rheumatoid arthritis
Retinol metabolism
Renin-angiotensin system
Proximal tubule bicarbonate reclamation
Protein processing in endoplasmic reticulum
Phagosome
Natural killer cell mediated cytotoxicity
Measles
Leishmaniasis
Graft-versus-host disease
Cytokine-cytokine receptor interaction
Complement and coagulation cascades
Breast cancer
Asthma
Ascorbate and aldarate metabolism
Allograft rejection
Aldosterone-regulated sodium reabsorption
Pathway
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Gene-number
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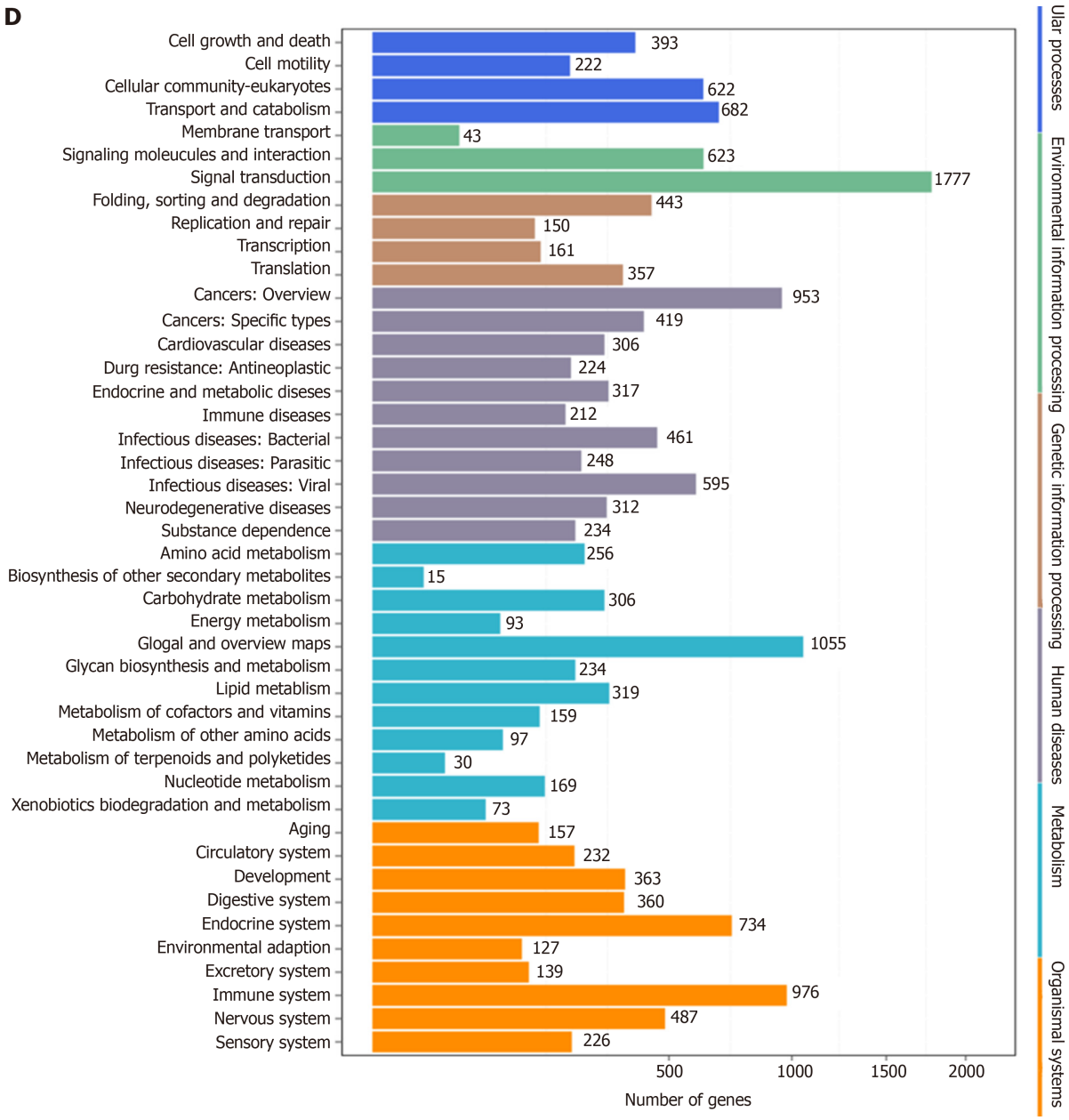
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Signal transduction 1777
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Infectious diseases: Parasitic 248
Infectious diseases: Viral 595
Neurodegenerative diseases 312
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Circulatory system 232
Development 363
Digestive system 360
Endocrine system 734
Environmental adaption 127
Excretory system 139
Immune system 976
Nervous system 487
Sensory system 226
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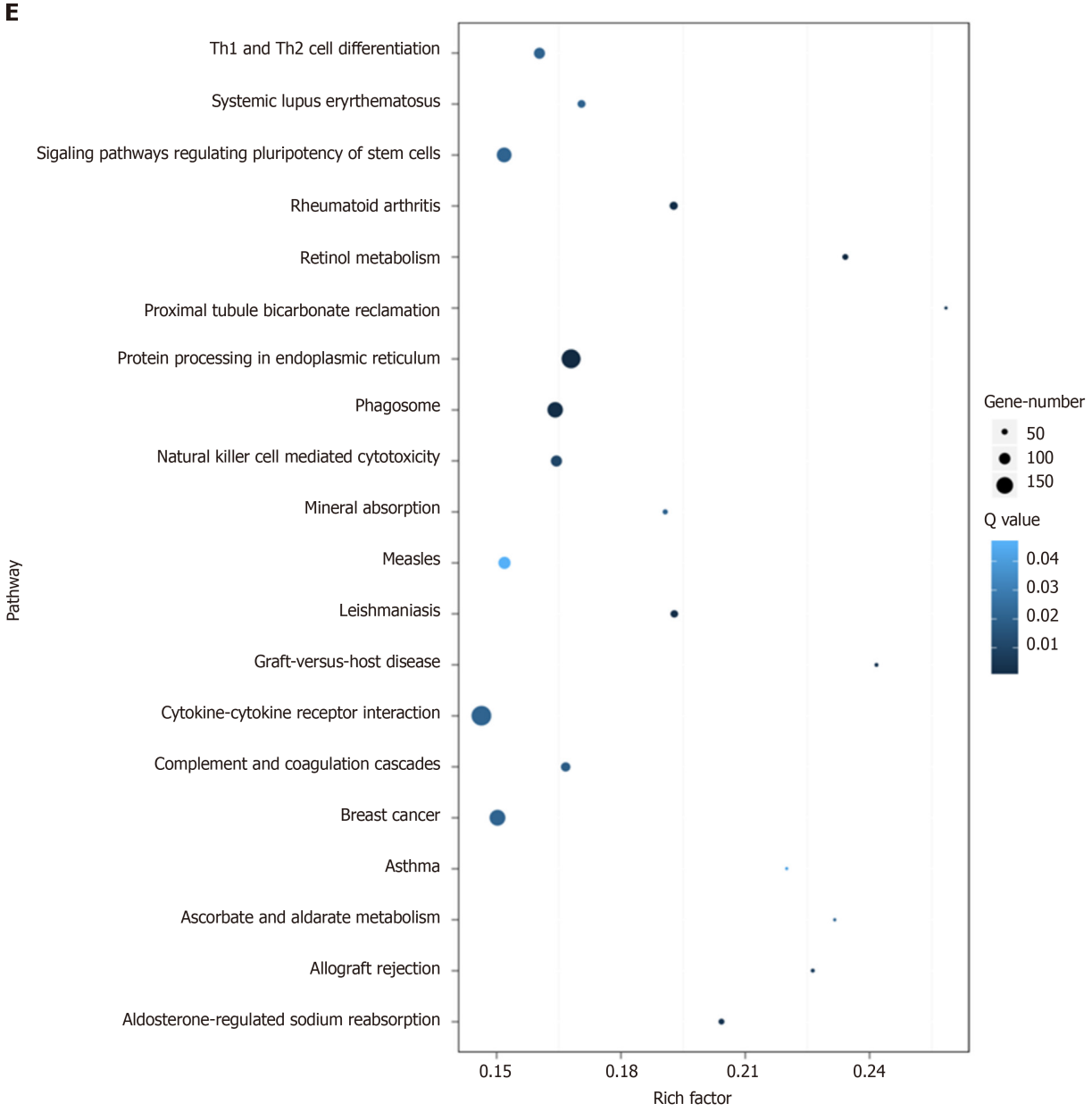
1000
1500
2000
Number of genes
Ular processes
Environmental information processing
Genetic information processing
Human diseases
Metabolism
Organismal systems

E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Sigaling pathways regulating pluripotency of stem cells
Rheumatoid arthritis
Retinol metabolism
Proximal tubule bicarbonate reclamation
Protein processing in endoplasmic reticulum
Phagosome
Natural killer cell mediated cytotoxicity
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Measles
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Graft-versus-host disease
Cytokine-cytokine receptor interaction
Complement and coagulation cascades
Breast cancer
Asthma
Ascorbate and aldarate metabolism
Allograft rejection
Aldosterone-regulated sodium reabsorption
Pathway
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Gene-number
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Q value
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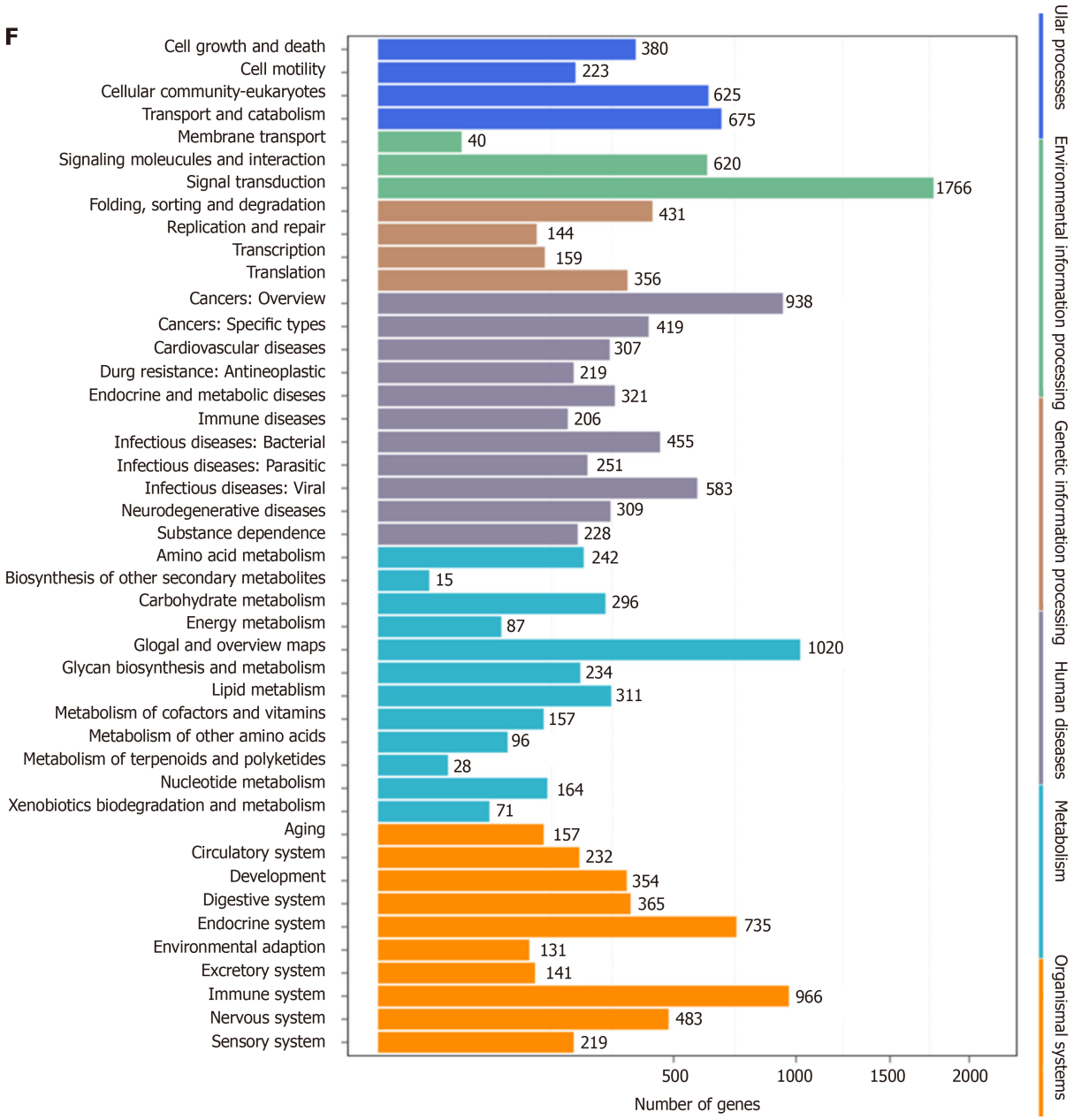
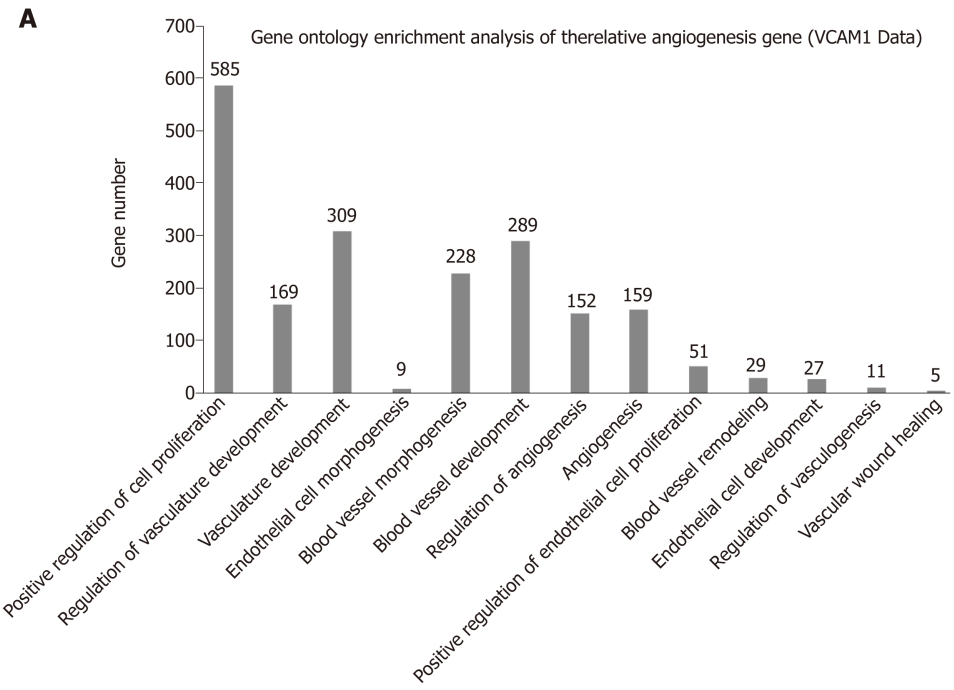


Figure 9 Pathway enrichment analysis of gene targets of differentially expressed miRNAs. A: Comparison of the top 20 enriched pathway terms between vascular cell adhesion molecule-1 (VCAM-1) group and control group; B: Comparison of the Kyoto Encyclopedia of Genes and Genomes (KEGG) classification between 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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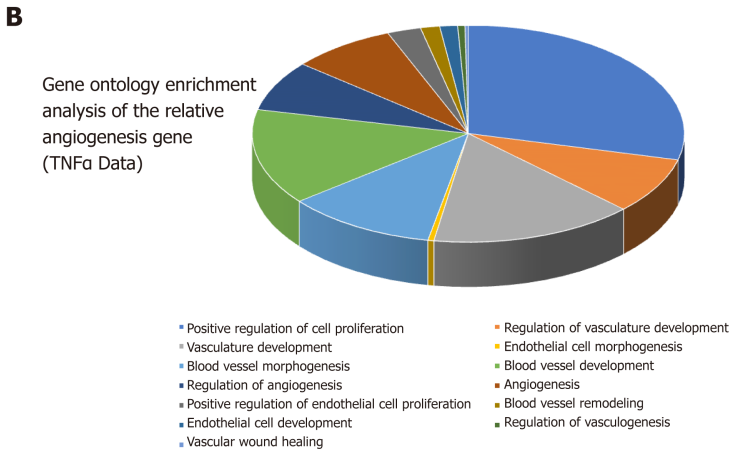
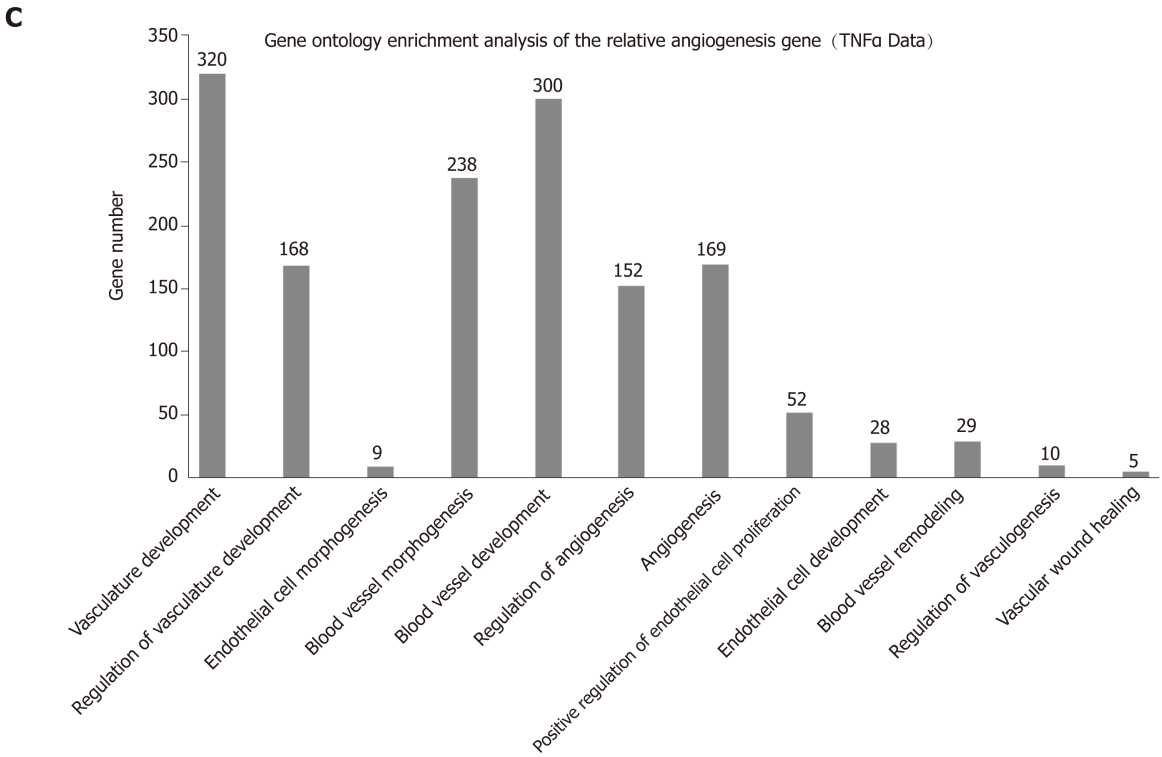
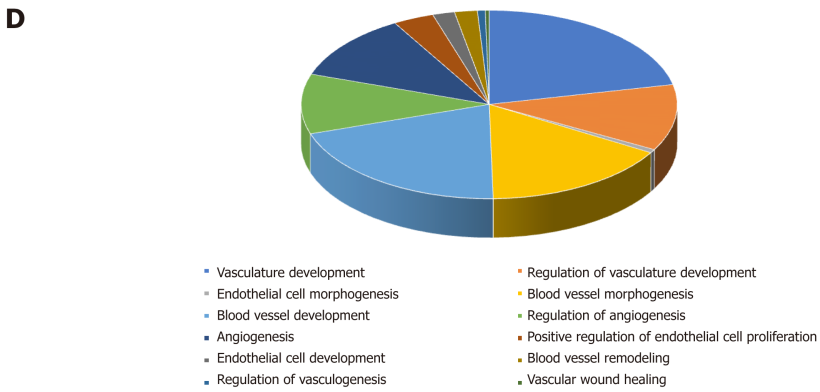
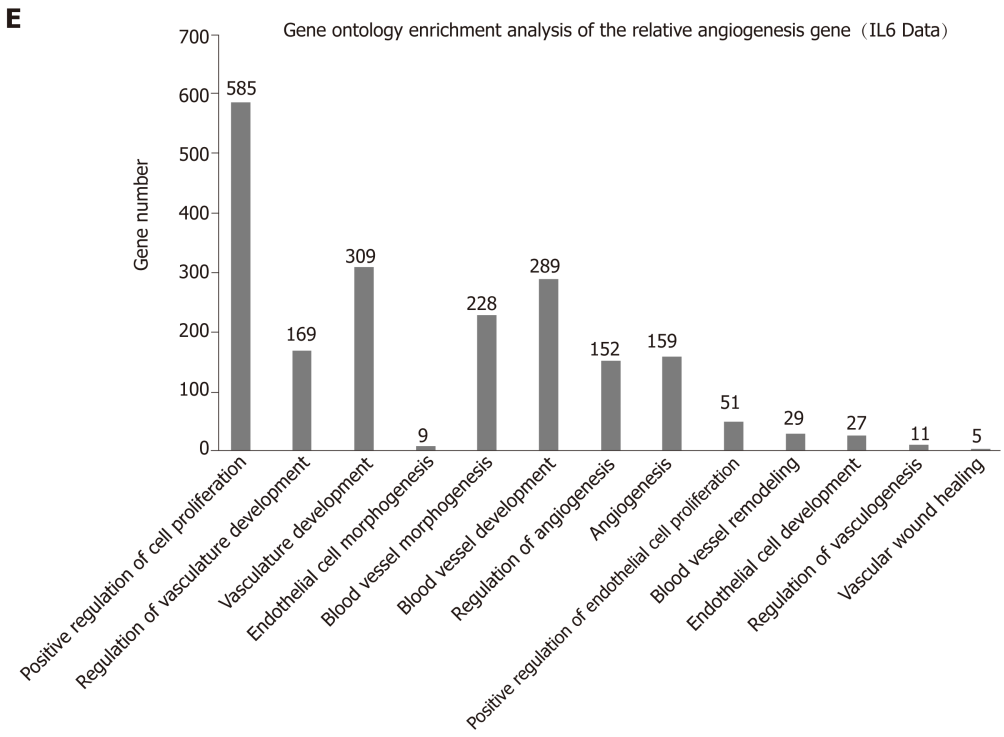
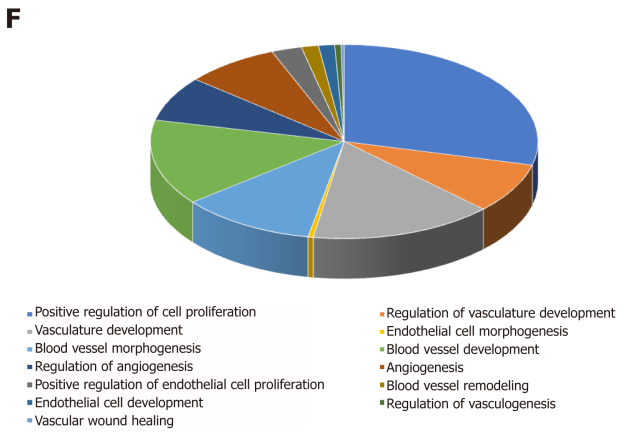
Gene ontology enrichment analysis of therelative angiogenesis gene (VCAM1 Data)
Gene number
700
600
500
400
300
200
100
0
585
169
309
9
228
289
152
159
51
29
27
11
5
Positive regulation of cell proliferation
Regulation of vasculature development
Vasculature development
Endothelial cell morphogenesis
Blood vessel morphogenesis
Blood vessel development
Regulation of angiogenesis
Angiogenesis
Positive regulation of endothelial cell proliferation
Blood vessel remodeling
Endothelial cell development
Regulation of vasculogenesis
Vascular wound healing

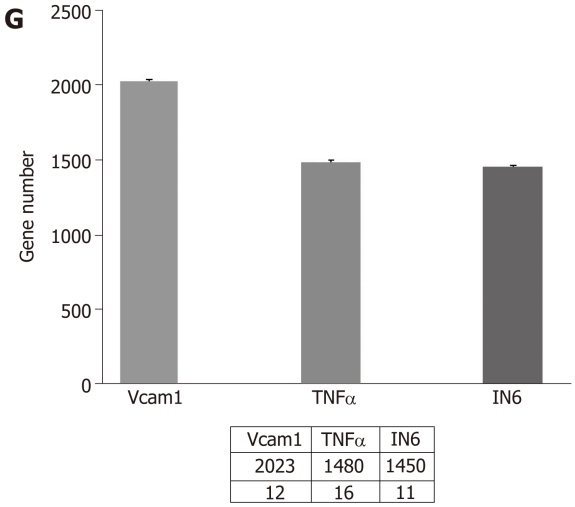

B
Gene ontology enrichment analysis of the relative angiogenesis gene (TNFα Data)
Positive regulation of cell proliferation
Regulation of vasculature development
Vasculature development
Endothelial cell morphogenesis
Blood vessel morphogenesis
Blood vessel development
Regulation of angiogenesis
Angiogenesis
Positive regulation of endothelial cell proliferation
Blood vessel remodeling
Endothelial cell development
Regulation of vasculogenesis
Vascular wound healing


C
Gene ontology enrichment analysis of the relative angiogenesis gene（TNFα Data）
Gene number
350
300
250
200
150
100
50
0
320
168
9
238
300
152
169
52
28
29
10
5
Vasculature development
Regulation of vasculature development
Endothelial cell morphogenesis
Blood vessel morphogenesis
Blood vessel development
Regulation of angiogenesis
Angiogenesis
Positive regulation of endothelial cell proliferation
Endothelial cell development
Blood vessel remodeling
Regulation of vasculogenesis
Vascular wound healing



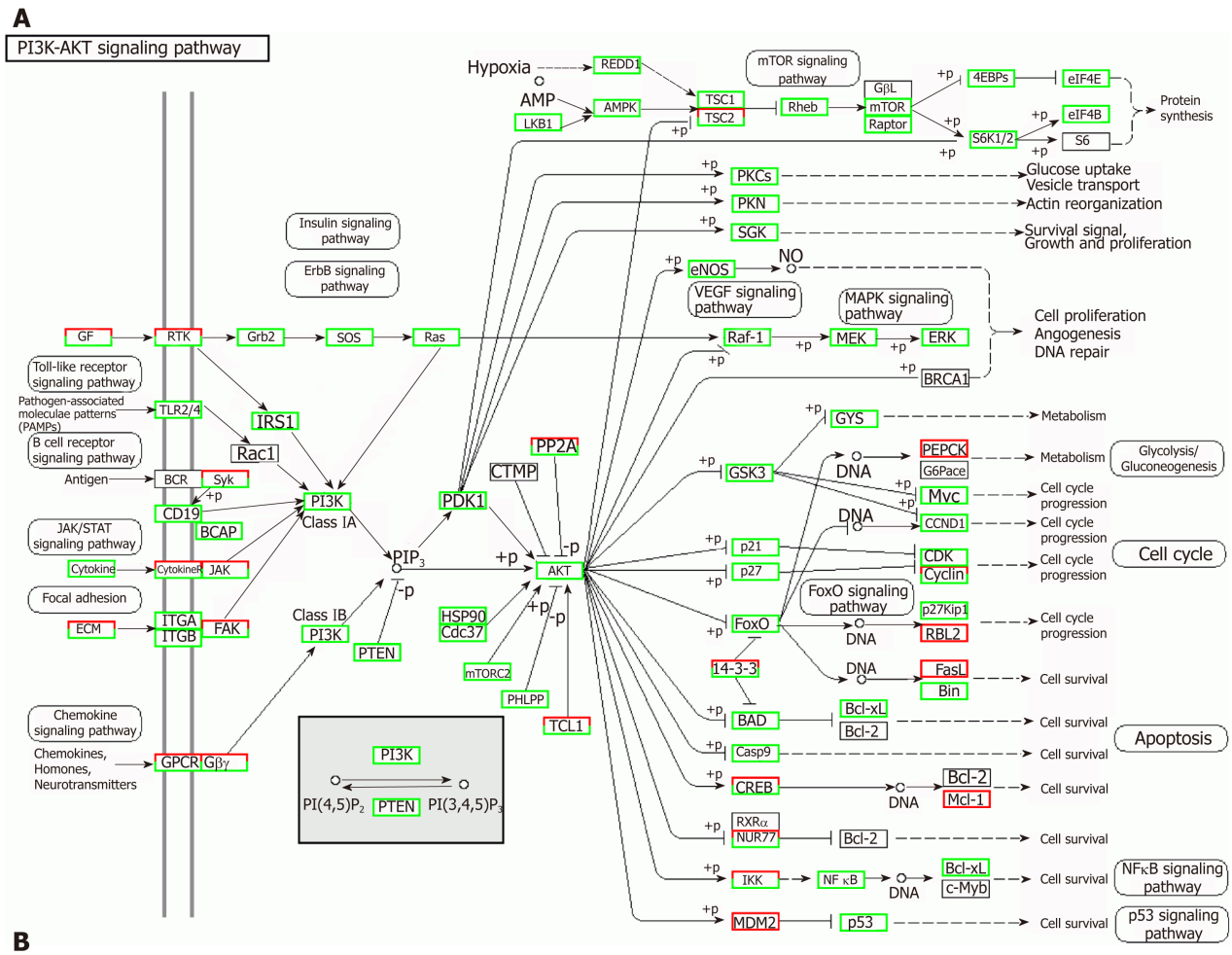






Vcam1	TNFα	IN6
2023	1480	1450
12	16	11

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.


A
PI3K-AKT signaling pathway
Hypoxia
REDD1
AMP
LKB1
AMPK
mTOR signaling pathway
TSC1
TSC2
Rheb
GβL
mTOR
Raptor
4EBPs
eIF4E
eIF4B
S6K1/2
S6
Protein synthesis
PKCs
PKN
SGK
Glucose uptake
Vesicle transport
Actin reorganization
Survival signal,
Growth and proliferation
Insulin signaling pathway
ErbB signaling pathway
eNOS
NO
VEGF signaling pathway
MAPK signaling pathway
GF
RTK
Grb2
SOS
Ras
Raf-1
MEK
ERK
BRCA1
Cell proliferation
Angogenesis
DNA repair
Toll-like receptor signaling pathway
Pathogen-associated moleculae patterns (PAMPs)
TLR2/4
IRS1
Rac1
B cell receptor signaling pathway
Antigen
BCR
Syk
CD19
BCAP
PI3K
Class IA
PIP3
PDK1
PP2A
CTMP
AKT
JAK/STAT signaling pathway
Cytokine
CytokineR
JAK
Focal adhesion
ECM
ITGA
ITGB
FAK
Class IB
PI3K
PTEN
HSP90
Cdc37
mTORC2
PHLPP
TCL1
GYS
Metabolism
GSK3
DNA
PEPCK
G6Pace
Glycolysis/
Gluconeogenesis
Myc
CCND1
Cell cycle progression
p21
p27
CDK
Cyclin
Cell cycle
FoxO signaling pathway
FoxO
p27Kip1
RBL2
14-3-3
FasL
Bin
Cell survival
BAD
Bcl-xL
Bcl-2
Apoptosis
Casp9
CREB
Mcl-1
RXRα
NUR77
IKK
NF κB
c-Myb
NFκB signaling pathway
MDM2
p53
p53 signaling pathway
Chemokine signaling pathway
Chemokines, Homones, Neurotransmitters
GPCR
Gβγ
PI3K
PI(4,5)P2
PTEN
PI(3,4,5)P3
B
PI3K-AKT signaling pathway

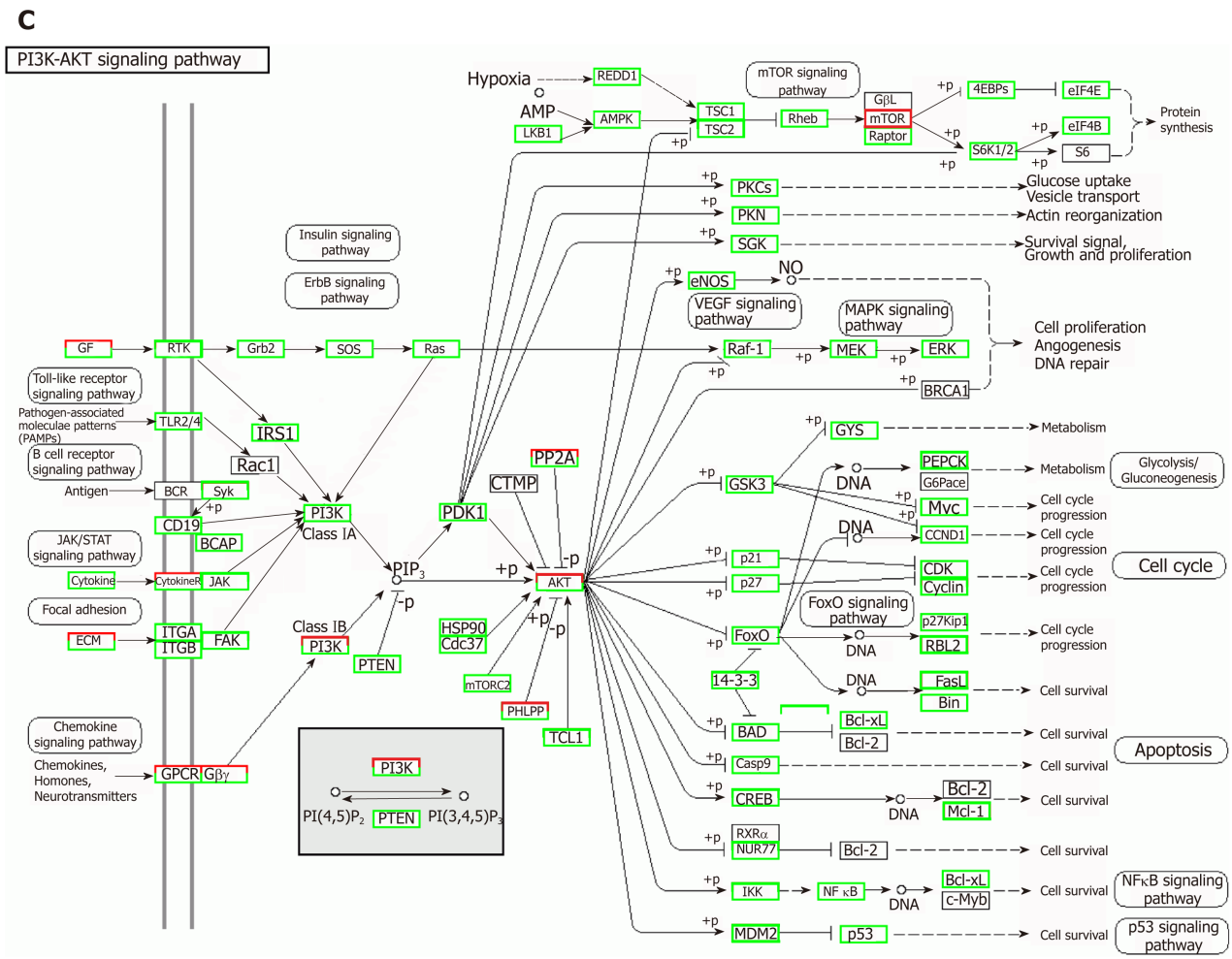
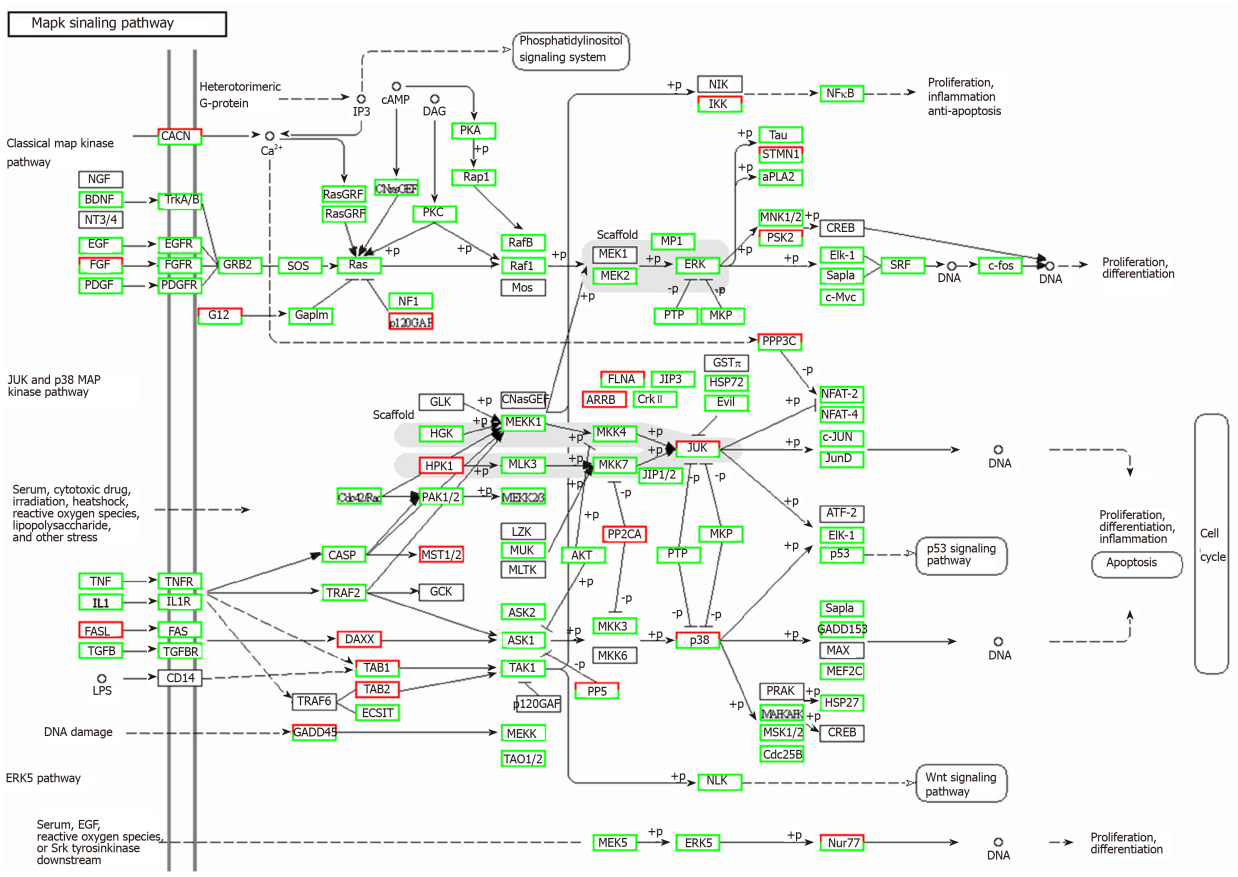
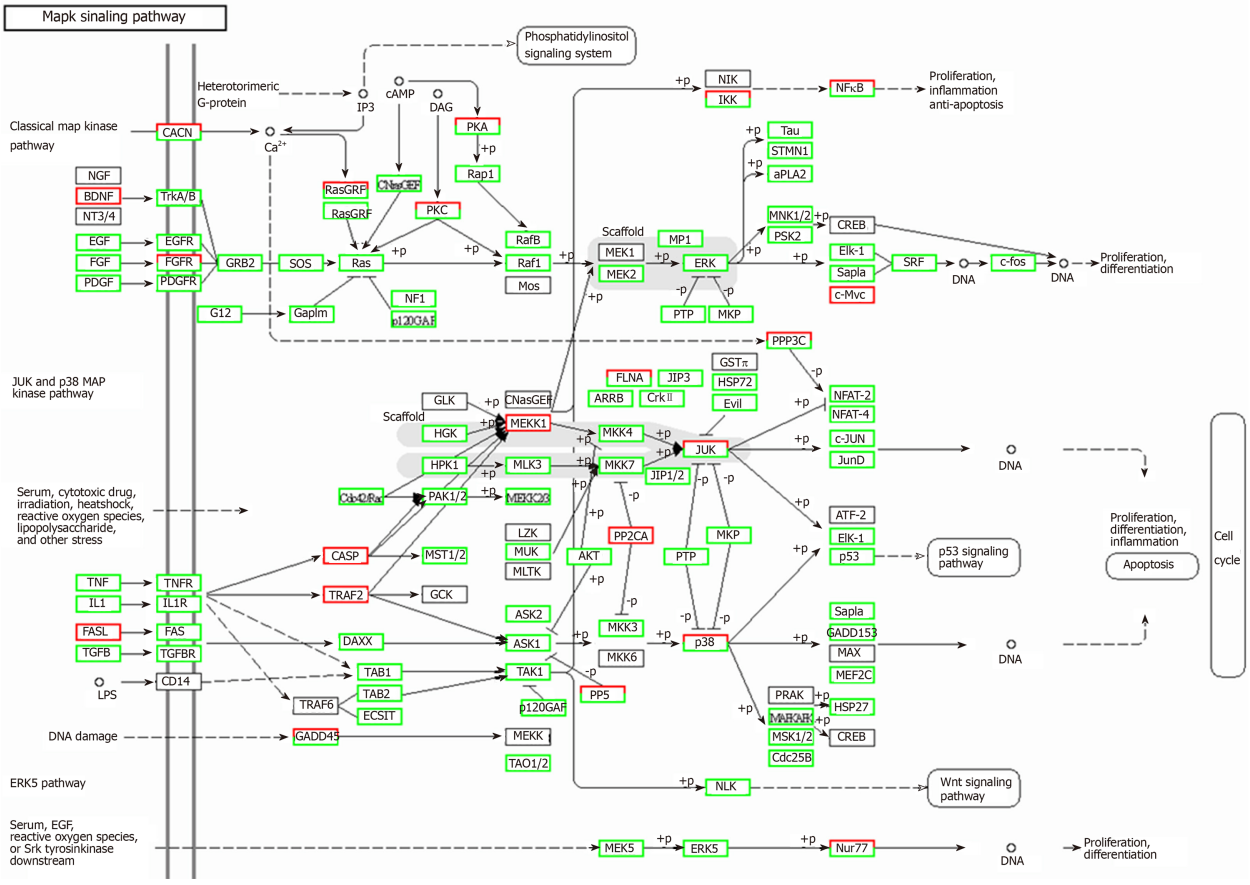


Figure 11 Regulatory mechanism of the PI3K-AKT signal pathway in different groups. A: Vascular cell adhesion molecule-1 group; B: Tumor necrosis factor α group; C: Interleukin 6 group. Up-regulated genes are marked with red borders and down-regulated genes with green borders. Unchanged genes are marked with black borders.

A


Mapk sinaling pathway
Phosphatidylinositol signaling system
Heterotorimeric G-protein
IP3
cAMP
DAG
Classical map kinase pathway
CACN
Ca2+
PKA
Rap1
RasGRF
PKC
NGF
BDNF
NT3/4
TrkA/B
EGF
FGF
PDGF
EGFR
FGFR
PDGFR
GRB2
SOS
Ras
RafB
Raf1
Mos
NF1
p120GAP
G12
Gaplm
Scaffold
MP1
MEK1
MEK2
ERK
PTP
MKP
NIK
IKK
NFκB
Proliferation, inflammation anti-apoptosis
Tau
STMN1
aPLA2
MNK1/2
PSK2
CREB
Elk-1
Sapla
c-Myc
SRF
DNA
c-fos
Proliferation, differentiation
PPP3C
JUK and p38 MAP kinase pathway
GSTπ
FLNA
JIP3
HSP72
ARRB
CrkII
Evil
GLK
HGK
HPK1
CNasGEF
MEKK1
MKK4
MKK7
MLK3
JUK
JIP1/2
NFAT-2
NFAT-4
c-JUN
JunD
Cdc42/Rac
PAK1/2
MEK3/6
LZK
MUK
MLTK
AKT
PP2CA
CASP
MST1/2
ATF-2
Elk-1
p53
p53 signaling pathway
Proliferation, differentiation, inflammation
Apoptosis
Cell cycle
Serum, cytotoxic drug, irradiation, heatshock, reactive oxygen species, lipopolysaccharide, and other stress
TNF
IL1
FASL
TGFB
TNFR
IL1R
FAS
TGFBR
CD14
LPS
TRAF2
GCK
ASK2
ASK1
DAXX
TAB1
TAB2
ECSIT
TAK1
TRAF6
PP5
MKK3
MKK6
p38
Sapla
GADD153
MAX
MEF2C
PRAK
MAPKAPK2
HSP27
MSK1/2
CREB
Cdc25B
DNA damage
GADD45
MEKK
TAO1/2
ERK5 pathway
NLK
Wnt signaling pathway
Serum, EGF, reactive oxygen species, or Src tyrosinkinase downstream
MEK5
ERK5
Nur77

B


Mapk sinaling pathway
Phosphatidylinositol signaling system
Heterotorimeric G-protein
Classical map kinase pathway
Scaffold
JUK and p38 MAP kinase pathway
ERK5 pathway
Serum, EGF, reactive oxygen species, or Src tyrosinkinase downstream
Proliferation, inflammation anti-apoptosis
Proliferation, differentiation
Proliferation, differentiation, inflammation
Apoptosis
Cell cycle
p53 signaling pathway
Wnt signaling pathway
DNA damage
Serum, cytotoxic drug, irradiation, heatshock, reactive oxygen species, lipopolysaccharide, and other stress

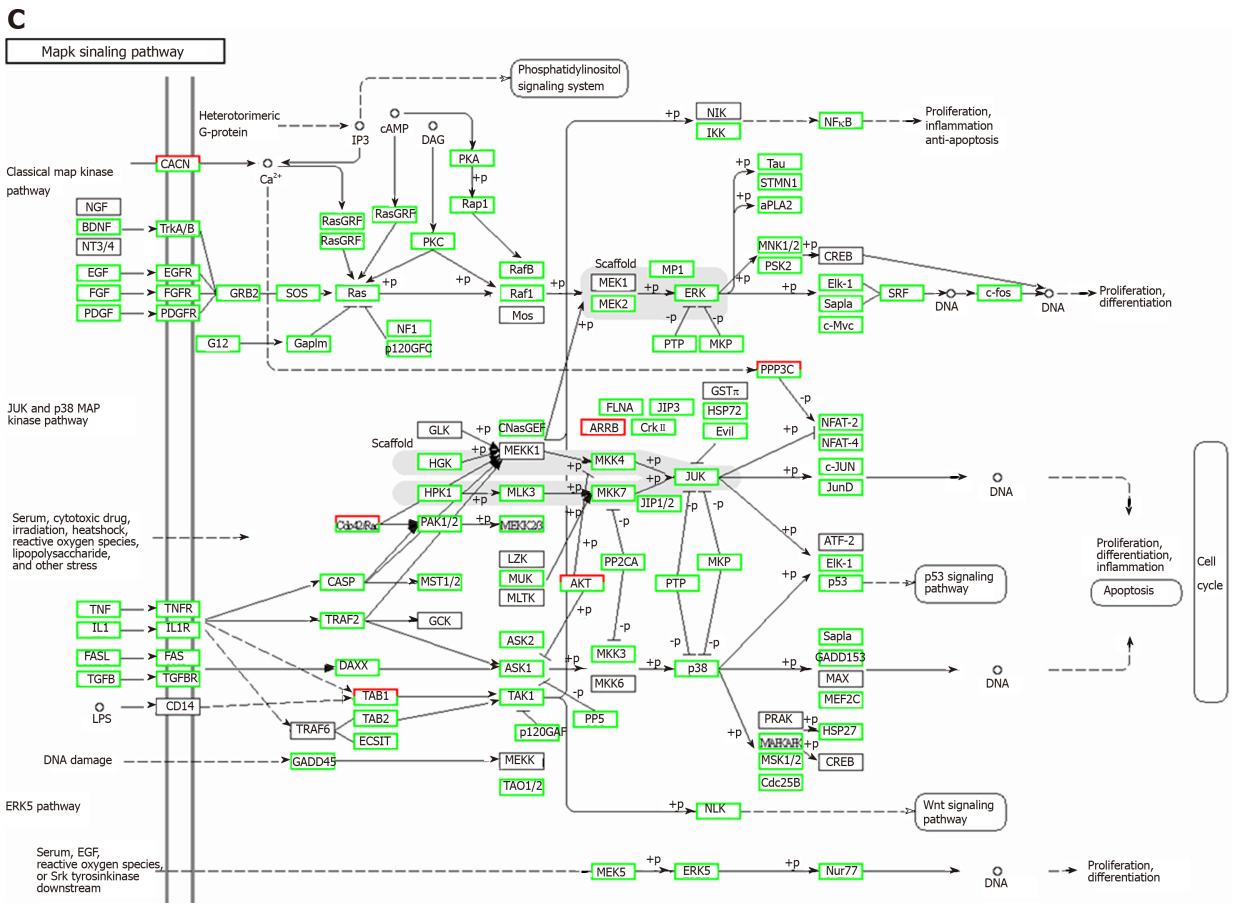


Figure 12 Regulatory mechanism of the MAPK signal pathway in different groups. A: Vascular cell adhesion molecule-1 group; B: Tumor necrosis factor α group; C: Interleukin 6 group. Up-regulated genes are marked with red borders and down-regulated genes with green borders. Unchanged genes are marked with black borders.

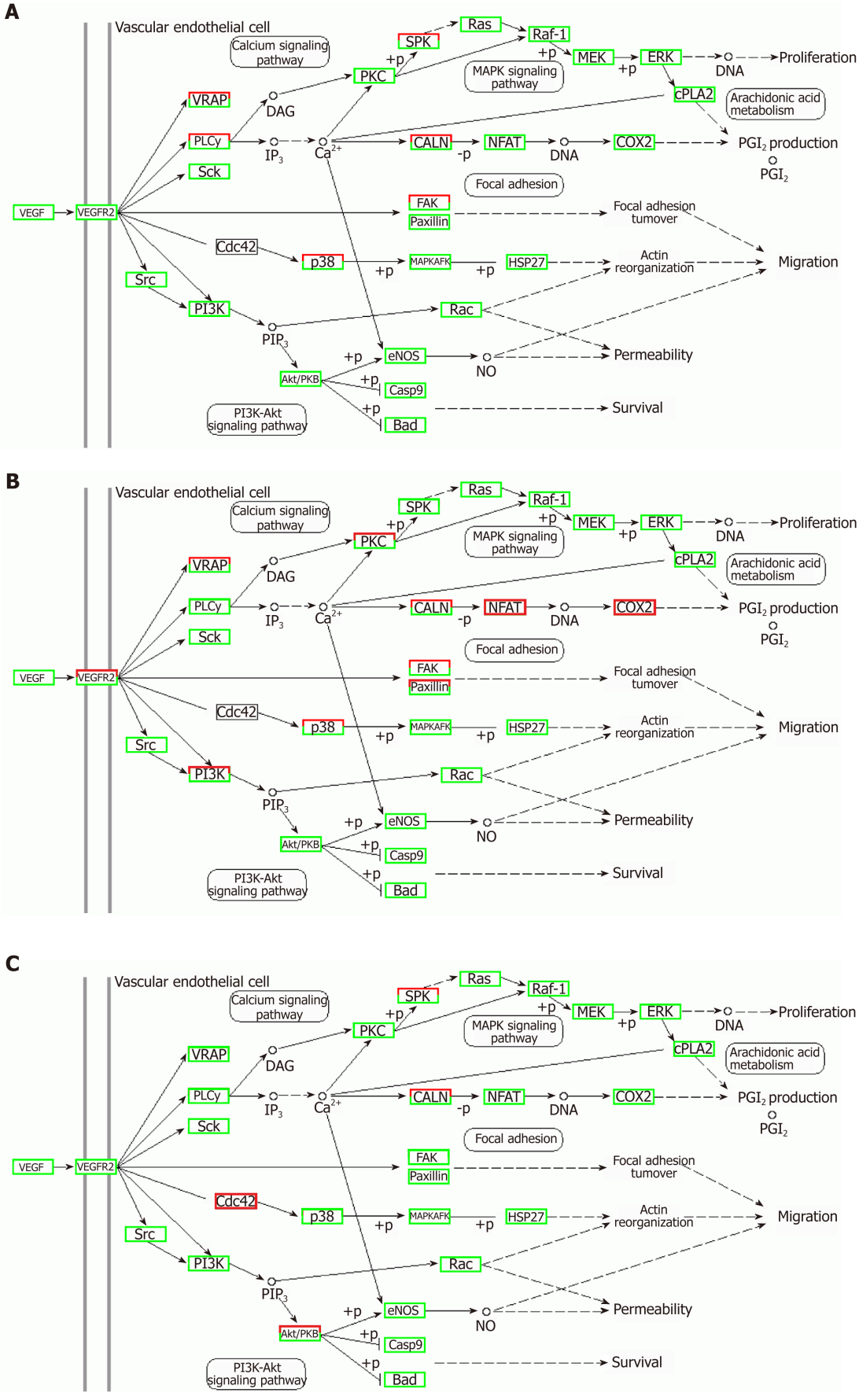


Figure 13 Regulatory mechanism of the VEGF signal pathway in different groups. A: Vascular cell adhesion molecule-1 group; B: Tumor necrosis factor α group; C: Interleukin 6 group. Up-regulated genes are marked with red borders and down-regulated genes with green borders. Unchanged genes are marked with black borders.

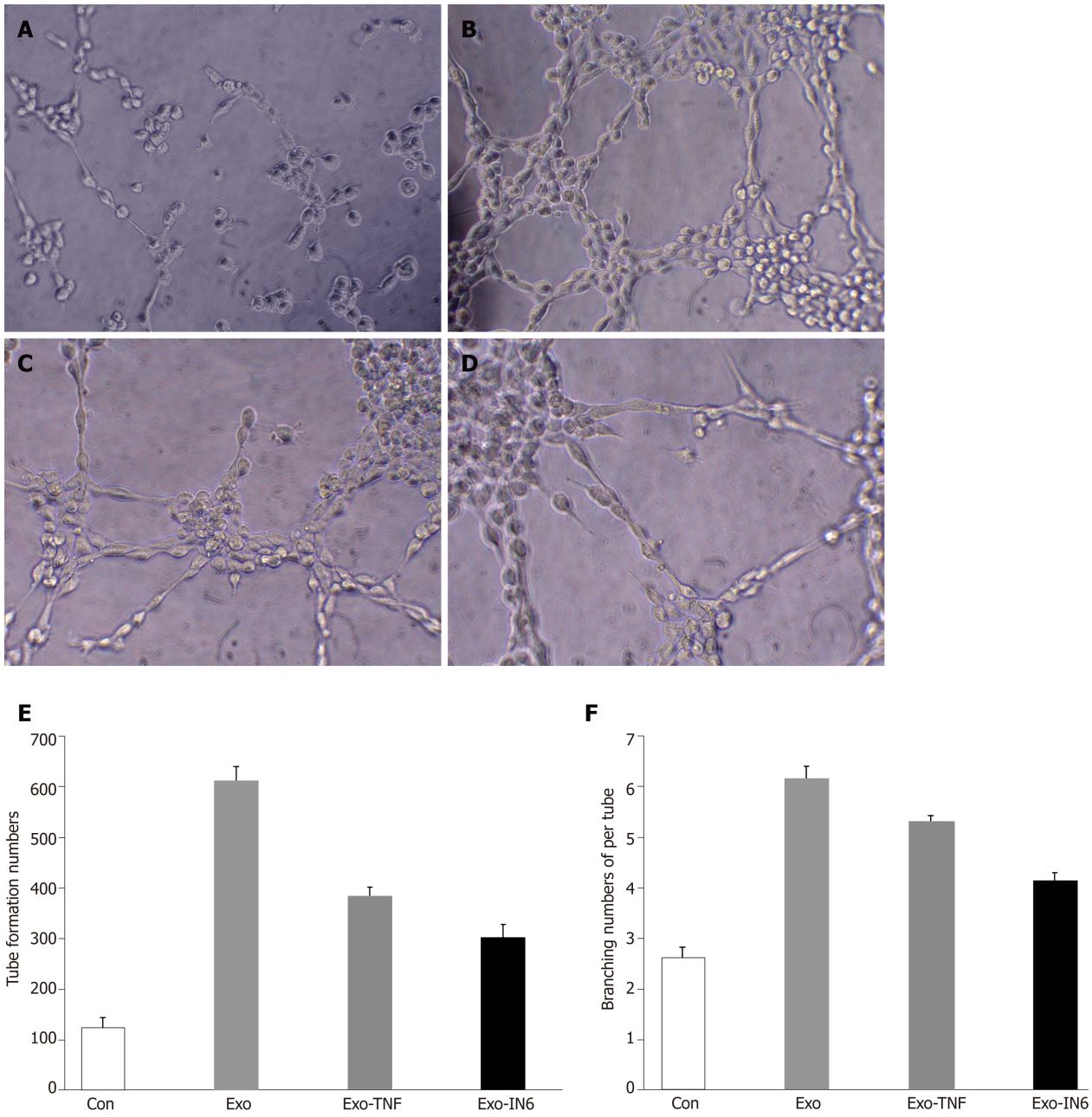


Figure 14 Tube formation in endothelial cells treated with mesenchymal stem cell exosomes. A: Micrograph showing human umbilical vein endothelial cells (HUVECs) cultured on Matrigel-coated plates in medium with phosphate buffered saline (control); B: Micrograph showing HUVECs cultured on Matrigel-coated 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.

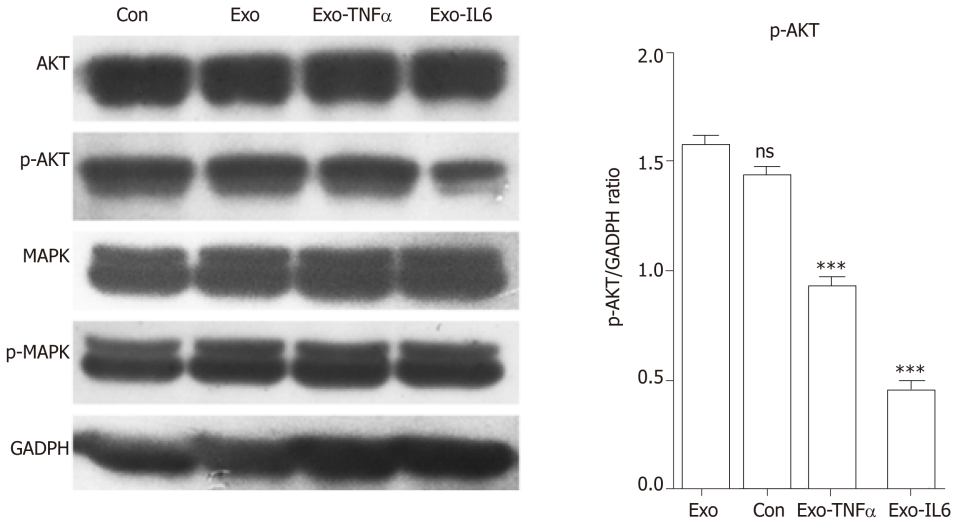


Figure 15 Western blot analysis. Western blot was performed to detect the expression of the indicated proteins in endothelial cells treated with phosphate buffered saline (control), mesenchymal stem cell exosomes (MSCs-exo), MSCs-exo$^{TNF\alpha}$ (stimulated with tumor necrosis factor α), MSCs-exo^{IL6} (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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