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Research Article

DOI:10.2478/rrlm-2022-0032

Endothelial progenitor cell (EPCs)-derived exosomal miR-30d-5p inhibits the inflammatory response of high glucose-impaired fibroblasts by affecting the M1/M2 polarization of macrophages

Wu Xiong[1], Mei-xin Tan[2], Zi-lin Chen[2], Yu Liu[3], Yang Liu[4], Xiao-ling Zou[5], Xiao-qin Wang[6], Ya Yang[6], Pei Tan[6], Xi Zhang[7*]

1. Department of Burns and Plastic Surgery, the First Affiliated Hospital of Hunan University of Chinese Medicine, China

2. College of Integrated Traditional Chinese and Western Medicine, Hunan University ofChinese Medicine, China

3. College of Traditional Chinese Medicine, Inner Mongolia Medical University, China

4. Department of plastic surgery, Xiangya Third Hospital, Central South University, China

5. Department of Endocrinolog, the First Affiliated Hospital of Hunan University of Chinese Medicine, China

6. College of Integrated Traditional Chinese and Western Medicine, Hunan University of Chinese Medicine, China

7. Hunan Brain Hospital (Clinical Medical School of Hunan University of Chinese Medicine), China

Abstract

***Background**: Diabetes is a common chronic disease which has caused a great burden on families and society. The treatment of diabetes has always been a hotspot. This study aimed to explore the effect and mechanism of miR-30d-5pon inflammation of high glucose-impaired human keloid fibroblasts (HKF). **Methods**: Differently-expressed miRNAs were predicted by bioinformatics methods. Exosomes were observed by transmission electron microscope. Exosome particle sizes were measured by NanoSight. Western Blot was used to detect the expression of CD81, CD63, CD9, and Calnexin. QRT-PCR was used to detect the expression of miR-30d-5p, IL-1β, TNF-α, VEGF, FGF21, NRF2, and HO-1. The levels of IL-1β, TNF-α, IL-6, IL-10, and TGF-β were determined by ELISA. Cell apoptosis and CD86, CD206 positive cells were detected by flow cytometry. **Results**: Tori formula could promote the secretion of endothelial progenitor cell (EPCs) exosomes. EPCs exosomes and miR-30d-5p could stimulate the*

* **Corresponding author**: Hunan Brain Hospital (Clinical Medical School of Hunan University of Chinese Medicine), China. E-mail: cmxfyjzx2049@126.com

proliferation of HKF impaired by high glucose and the expression of IL-10 and TGF-β. MiR-30d-5p inhibited the proliferation of M1 macrophages and the expression of IL-1β and TNF-α. It could also promote the proliferation of M2 macrophages and the expression of CCL17 and CCL22. Moreover, miR-30d-5p stimulated the expression of VEGF, FGF21, NRF2, and HO-1, as well as suppressed the expression of IL-1β, TNF-α, and IL-6. MiR-30d-5p also restrained the apoptosis of impaired HKF. ***Conclusion****: This study confirmed that miR-30d-5p could promote the M1/M2 polarization and inhibit the inflammatory response of impaired HKF, which provided a certain idea and direction for treating diabetes.*

Keywords*: diabetes mellitus, miR-30d-5p, high-glucose impaired HKF, EPCs exosomes*

Received: 12th May 2022; Accepted: 25th June 2022; Published: 16th August 2022

Introduction

Diabetes Mellitus (DM) is a common disease. Patients usually suffer symptoms such as polydipsia, polyuria, and weight loss. It is often accompanied by obesity, high glucose, and high blood lipids. The prevalence rate is rapidly increasing yearly, and it is estimated that 592 million people will be affected by 2035 (1). In addition, because many DM patients do not show obvious symptoms at an early stage, it is considered that the actual number of patients should be higher than the number of those confirmed (2). Diagnosis of DM is often accompanied by serious complications. Delays in treatment often lead to complications such as refractory wounds and diabetic feet, which are also important factors of death and disability in diabetic patients (3). Studies have shown that diabetes and some cardiovascular diseases are accompanied by high expression of inflammatory factors (4). Inflammatory factors are secreted by different cells and regulate different tissues through systemic circulation (5). The disease pathway is also considered a common pathogenic mediator of diabetes, overweight and cardiovascular disease (6), and lots of clinical chronic inflammation is also caused by the natural pathogenesis of DM and its complications (7). However, diabetes and a series of complications have complex pathogenic factors, and there is no effective treatment, which burdens diabetes patients and medical institutions greatly.

Macrophages play an important role in the progression of inflammatory diseases including DM (8). Macrophages are typical immune cells that could react to the corresponding microenvironmental stimuli and signals to form specific phenotypes, that is, polarization (9). According to the expression of macrophage surface markers and the biological activity of specific factors, several types of macrophages have been reported in humans. The most representative ones are inflammation-related M1 type and anti-inflammatory-related M2 type (10-12). M1 macrophages are often induced by Th1 cytokines such as TNF-α. The induced cells will secrete more TNF-α, IL-1α, IL-1β, IL-6 and lower levels of IL-10. M1 macrophages have strong anti-microbial and anti-tumor activities, which could promote ROS-induced tissue damage, damaged tissue regeneration and wound healing. The anti-inflammatory function regulation of M2 macrophages could inhibit this inflammatory response and relevant damage in human bodies (12-14). M2 macrophages could be polarized by IL-4, IL-10 and secrete high levels of anti-inflammatory factors such as IL-10 and TGF-β (15), which is characterized by the up-regulation of CCL17, CCL24 and other chemokines (16). The secreted IL-10 and TGF-β have the properties of promoting angiogenesis and fibrosis (17). MicroRNAs (miRNAs) are a class of single-stranded non-coding RNA about 19-25 nucleotides in length, which often act as regulators

of gene expression by binding to the 3'UTR of target mRNA (18-20). Many exosome-derived microRNAs are involved in regulating metabolic disorders. Studies have shown that exosomal miR-21-5p, miR-375-3p, miR-451-5p, let-7C-5p and miR-362-3p are up-regulated in serum and urine of diabetic patients (20, 21), suggesting that some exosome miRNAs can participate in the regulation of diabetes. MiR-30d-5p was initially identified as a tumor suppressor miRNA, which plays a key role in controlling the proliferation, migration, and invasion of tumor cells (22). Research has revealed that miR-30d-5p could regulate Caspase-1 activation and promote the release of IL-1β and IL-18 (23), which is also related to the severity of systemic inflammatory response syndrome and the steady-state level of redox in vivo, suggesting that miR-30d-5p may be a potential regulator of inflammation (24). However, no studies have reported the role and mechanism of miR-30d-5p in hyperglycemic inflammatory cells.

In this study, differential expressions of miR-30d-5p in different blood glucose samples were screened out through bioinformatics prediction. Then, related genes in high-glucose impaired human keloid fibroblasts (HKF) and different types of macrophages were detected to explore the regulatory mechanism of miR-30d-5p on inflammatory response in HKF. This study provided a new idea and direction for treating diabetes, which was of great significance.

Materials and methods

Cell acquisition and processing

Endothelial progenitor cells (EPCs) were obtained from the laboratory during previous studies, and our study passed the ethics review of the First Affiliated Hospital of Hunan Chinese Medicine University (HN-LL-KY-2020-013-01). Human monocytes (THP-1) were purchased from Wuhan Procell (CL-0233, Procell, China), and human keloid fibroblasts (HKF) were purchased from FENGHUI (CL0338, FENGHUI, China). EPCs were cultured in DMEM (Dulbecco's Modified Eagle Medium) at 37℃, 5% CO_2, added with 20% Fetal Bovine Serum (FBS), 105 mg/L penicillin and 100 mg/L streptomycin. THP-1 cells were induced at 37℃, 5% CO_2 for 24 h in RPMI 1640 medium for differentiation into macrophages. Subsequently, the cells were cultured in Iscove modified Dulbecco medium (IMDM, Life Technologies) at the concentration of 1×10^6 /mL with 10% Fetal Calf Serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin and 25 ng/mL human macrophage stimulating factor (M-CSF, Miltenyi). HKF cells were cultured in DMEM medium containing 5% phosphate buffered saline (PBS) and 1% penicillin-streptomycin at 37℃, 5% CO_2.

In order to explore the promoting effect of tori disinfection compound on miR-30d-5p, tori disinfection compounds (ferulic acid, astragaloside, and ginsenoside were dissolved in media with the concentrations of 8 mg/L, 100 mg/L and 40 mg/L, which is optimal for usage in the early study of lab) were used to deal with the EPCs. The exosomes secreted by EPCs were divided into the following 4 groups: Control group (normal EPCs exosomes), L-TUO group (EPCs were treated with 50% optimal concentration of tori disinfection compound for 48 h before exosomes were collected), M-TUO group (EPCs were treated with the optimal concentration of tori disinfection compound for 48 h before exosomes were collected), H-TUO group (EPCs were treated with 2 times optimal concentration of tori disinfection compound for 48 h before exosomes were collected).

In order to explore the effect of miR-30d-5p on HKF impaired by high glucose, HKF with different treatments were divided into the following three groups: Control group (HKF were cultured in DMEM), H-Glucose group (HKF were cultured in 30 mM glucose medium (25)), H-Glu-

cose+Exosomes group (HKF were cultured in 30 mM glucose medium added with exosomes from M-TUO group).

To verify the transfection effect of miR-30d-5p in macrophages, macrophages were divided into the following two groups: exo-NC group (macrophages were co-cultured with EPCs transfected with mimics NC for 48 h after stimulated by LPS), exo-miR-30d-5p mimics group (macrophages were co-cultured with EPCs transfected with miR-30d-5p mimics for 48 h after stimulated by LPS).

To investigate the effect of miR-30d-5p on the polarization of macrophages from M1 to M2, macrophages with different treatments were divided into the following four groups based on M1/M2: Control group (untreated macrophages), LPS/IL-4 group (macrophages were stimulated by 1 μg/mL LPS/IL-4), LPS+NC/IL-4+NC group (macrophages were stimulated by 1 μg/mL LPS/ IL-4, and exosomes secreted by EPCs transfection with mimics NC were added), LPS+miR-30d-5p mimics/IL-4+miR-30d-5p mimics group (macrophages were stimulated by 1 μg/mL LPS/IL-4, and exosomes secreted by EPCs transfection with miR-30d-5p mimics were added).

To verify the role of miR-30d-5p in HKF, HKF were divided into the following four groups: mimics NC group (HKF were cultured with a high concentration of 33 mM glucose, and exosome secreted by EPCs transfected with mimics NC were added), exo-miR-30d-5p mimics group (HKF were cultured with a high concentration of 33 mM glucose, exosome secreted by EPCs transfected with miR-30d-5p mimics were added), inhibitor NC group (HKF were cultured with high concentration of 33 mM glucose, exosome secreted by EPCs transfected with inhibitor NC were added), miR-30d-5p inhibitor group (HKF were cultured with a high concentration of 33 mM glucose, exosomes secreted by EPCs transfection with miR-30d-5p-silenced plasmid were added).

Cells transfection

MiR-30d-5p mimics, mimics NC, inhibitor NC and miR-30d-5p-silenced plasmid were synthesized by GenePharma (Shanghai, China). Lipofectamine 2000 reagent (Invitrogen, USA) was used to take the transfection according to instructions.

Quantitative real-time PCR (qRT-PCR)

Tissue cells stored in the precooled Trizol (15596026, ThermoFisher, USA) were taken, RNA was extracted with Trizol, then the spectrophotometer was used to measure RNA concentration. The reverse transcription kit (CW2569 CWBIO, China) was used to prepare the reverse transcription system and carried out transfection according to the instructions. Using cDNA as a template, UltraSYBR Mixture (CW2601, CWBIO, China) was taken for qRT-PCR. The instrument was from ThermoFisher (PIKOREAL96, ThermoFisher, USA). The primer sequences are shown in Table 1, β-actin and U6 were used as internal reference genes, and relative expression levels were calculated using $2^{-\Delta\Delta Ct}$ method.

Western Blot

After the sample cells were washed with PBS, RIPA lysate (AWB0136b, abiowell, China) was added to lysate the cells. The lysis fluid was transferred to a table top refrigerated centrifuge (H1650R, XIANGYI, China) for centrifugation, and the protein concentration of the supernatant was determined. Electrophoresis was performed after denatured in boiling water bath. Afterwards, the membrane transfection was done with a constant current in a membrane converter (DY-CZ-40D, LIUYI, China). Skim milk powder/BSA (YanchengSaibao, China) of 5% was prepared, the sample was placed at room temperature for 1 h and sealed at 4°C overnight. Then

Table 1. Primer sequences used in qRT-PCR

Name	Sequences
β-actin	Forward ACCCTGAAGTACCCCATCGAG
	Reverse AGCACAGCCTGGATAGCAAC
U6	Forward CTCGCTTCGGCAGCACA
	Reverse AACGCTTCACGAATTTGCGT
miR-30d-5p	Forward GTTGTTGTAAACATCCCCGACTG
	Reverse GTAGCAGCAAACATCTGACTGA
IL-1β	Forward CCCTCTGTCATTCGCTCCC
	Reverse TAAAGAGAGCACACCAGTCCA
TNF-α	Forward GAACCCCGAGTGACAAGCCT
	Reverse TATCTCTCAGCTCCACGCCAT
CCL17	Forward GAATTCAAAACCAGGGTGTCTCC
	Reverse CACATTGGTCCCTCGAGCTG
CCL22	Forward GAGATCTGTGCCGATCCCAG
	Reverse AGGGAATGCAGAGAGTTGGC
VEGFA	Forward TGCTCTACTTCCCCAAATCACT
	Reverse ACTCACTTTGCCCCTGTCG
FGF21	Forward TCCCTCCGACTCAGACCCAG
	Reverse CGCCTGAACCATGATATTTGACC
NRF2	Forward CAACTCAGCACCTTATATCTCG
	Reverse ACAAGGAAAACATTGCCATC
HO-1	Forward CACACCCAGGCAGAGAATGCT
	Reverse GGCTCTCCTTGTTGCGCTCA

the membrane was incubated with first antibodies, including CD81 (ab109201, 1:5000, Abcam, UK), CD63 (25682-1-AP, 1:1000, Proteintech, USA), CD9 (ab236630, 1:1000, Abcam, UK), Calnexin (ab133615, 1:3000, Abcam, UK), and internal reference β-actin (66009-1-Ig,1:5000, proteintech, USA), and placed at room temperature for 30 min the following day. After incubation, the diluted secondary antibody was incubated with the membrane at room temperature for 90 min after being cleaned with PBST (PBS added with 0.05% Tween 20) 3 times. The secondary antibody included HRP goat anti-mouse IgG (SA00001-1, 1:5000, Proteintech, USA), HRP goat anti-rabbit IgG (SA00001-2, 1:6000, Proteintech, USA). After incubation, ECL chemical luminescent liquid was used for development, and the gel imaging system was used for imaging.

Apoptosis detection

HKF and macrophages of different treatments were collected with trypsin digestion solution (C0201, Beyotime, China). The samples were washed by phosphate buffer solution (PBS) (SH30256.01, Hyclone, China). Then, the cells were centrifuged and collected. The binding buffer was added to suspend cells and 5 μL Annexin V-APC was added (KGA1030, KeyGEN, China). After mixing, 5 μL Propidium Iodide was added for reaction at room temperature under dark conditions for 10 min. Apoptosis was observed by flow cytometer within 1 h.

Flow cytometry

Cells to be tested were transferred into 1.5 mL EP tubes, PBS was added to wash the cells, and the supernatant was discarded after centrifugation. The same procedure was repeated. CD86 Monoclonal Antibody (MA1-10295, eBiosci-

ences, USA) and CD206 antibody Anti-Mannose Receptor (ab270647, eBiosciences, USA) were added into each tube. After being dyed for 30 min at room temperature under dark conditions, PBS was added to wash cells, the supernatant was discarded by centrifugation, and PBS was added again to re-suspend the precipitation, finally the cells were observed by flow cytometer.

Cell counting kit-8 (CCK-8) assays

HKF were incubated in a 96-well plate (0030730119, Eppendorf, Germany) with a density of 5×10^3 cells/well, 100 μL per well with 4 repeat wells in each group. The cells were cultured at 37°C for 12 h, 24 h, and 48 h under dark conditions. After 10 μLCCK-8 (NU679, Tonen, Japan) was added, HKF were cultured at 37°C, 5% CO_2 for 4 h. Then Bio-Tek microplate analyzer (MB-530, Heales, China) was used to analyze the absorbance at 450 nm. The average value was taken to calculate the growth inhibition rate.

Enzyme linked immunosorbent assay (ELISA)

IL-6 ELISA kit (CSB-E04638h, CUSABIO, China), TNF-α ELISA kit (CSB-E04740h, CUSABIO, China), TGF-β1 ELISA kit (CSB-E04725h, CUSABIO, China), IL-1β ELISA kit (CSB-E08053h, CUSABIO, China), IL-10 kit (CSB-E04593h, CUSABIO, China), and MCP-1 ELISA kit (CSB-E04655h, CUSABIO, China) were used to detect the levels of each inflammatory factor. The optical density (OD) of each well was measured in sequence at 450 nm with a multifunctional enzyme label analyzer (DHP-500, Everbright, China) within 5 min after the termination of the reaction.

Bioinformatics analysis

Nine diabetic hyperglycemic samples and 10 normal blood glucose samples were screened from the GSE21321 dataset. R language limma package was used to analyze the differentially expressed miRNAs, and the volcanic map of miRNAs with significant differences between hyperglycemia and normal samples was plotted. Then, four miRNAs with significant differences between hyperglycemia and normal samples, as well as lower expression in hyperglycemia samples, were selected to draw the box plot. After miR-30d-5p was screened, the Cluster Profiler package of R language was used for target prediction and enrichment analysis. Finally, the immune pathway mediated by miR-30d-5p was predicted and analyzed through GO and KEGG.

Extraction and electron microscopic observation of exosomes

EPCs cell fluid was centrifuged to remove cell debris, and the supernatant was transferred to a clean sterilization tube. EPCs exosomes were extracted according to the product instructions of ExoQuick (SBI, EXOQ5A-1, USA). Subsequently, 100 μL exosomes were prepared, the coated copper net was placed into the exosomes sample for 2 min, after washed with distilled water, fixed with 2.5% glutaraldehyde for 2 min. Then, the samples were stained with 2% uranium acetate for 2 min. After the copper mesh was cleaned and dried, the images were observed under transmission electron microscopy (JEM1400, JEOL, Japan). The images were recorded by a digital camera.

Nanoparticle tracking analysis (NTA)

NTA was performed on an LM10-HS microscope with NTA Software V2.3 (NanoSight, UK). The minimum expected particle radius and track length were set according to system settings. The diluted samples were recorded three times at 30 frames per second, and the complete track measurement results were analyzed.

Data processing and analysis

GraphPad Prism 8.0 statistical software was used to process the obtained data. One-way ANOVA

and unpaired sample *t* test were used to analyze the significance of differences between the calculated data. It is considered statistically significant when $p<0.05$.

Results

Differentially expressed miRNAs in diabetes were predicted by bioinformatics

MiRNAs with differential expression in hyperglycemia and normal samples were screened, hsa-miR-144-5p, hsa-miR-720, hsa-miR-92a-3p, hsa-miR-30d-5p, hsa-miR-192-5p, hsa-miR-29a-3p, hsa-miR-320a-3p, and hsa-miR-144-3p were eight miRNAs selected with different expression (Figure 1A). Of these miRNAs, hsa-miR-144-5p, hsa-miR-720, hsa-miR-92a-3p, and hsa-miR-30d-5p showed different expressing trends between hyperglycemia and normal samples (Figure 1B). Finally, hsa-miR-30d-5p was selected for further exploration. The immune pathway mediated by miR-30d-5p was predicted through GO and KEGG databases (Figure 1C).

Tori formula promoted the secretion of exosome miR-30d-5p by endothelial progenitor cells (EPCs)

The exosomes of the four groups were observed under a transmission microscope. It could be found that the number of exosomes in the tori formula-treated group was significantly higher, and M-TUO showed more exosomes than H-TUO and L-TUO (Figure 2A). These results indicated that the tori formula could promote EPCs secretion of exosomes, and the promotion effect was relatively better at a more appropriate concentration (the concentration in M-TUO). NTA showed that the particle number of M-TUO group was significantly increased compared with that of L-TUO and H-TUO groups, and all three groups had more particles than the Control group (Figure 2B). This data proved that the appropriate concentration of tori formula had a better promoting effect on the secretion of exosomes. The expression levels of CD9, CD63, and CD81 in the Control group and M-TUO group were detected, and the results demonstrated that CD9, CD63, and CD81 were expressed in exosomes of both groups (Figure 2C), indicating successful identification of exosomes. In addition, qRT-PCR revealed that the expression level of miR-30d-5p in M-TUO group was higher than that of L-TUO and H-TUO, the expression level of miR-30d-5p in exosomes of these 3 groups was significantly higher than that of the Control group (Figure 2D). These results showed a similar trend to the number of exosomes, suggesting that the tori formula could also promote the expression of miR-30d-5p.

EPCs-derived exosomes promoted the proliferation of high-glucose impaired HKF and inhibited inflammation

CCK-8 analysis showed that the cell viability of the H-Glucose group was significantly inhibited compared with that of the Control group, reflecting the inhibitory effect of high glucose on cell viability. Compared with the H-Glucose group, the cell viability of the H-Glucose+Exosomes group increased significantly (Figure 3A), indicating that EPCs exosomes could promote the proliferation of HKF cells with high glucose impairment. To further verify the results, flow cytometry showed that the apoptosis level of cells in the H-Glucose group was higher than that of the Control group, while the apoptosis rate in H-Glucose+Exosomes group was significantly lower than that in H-Glucose group (Figure 3B). This was consistent with CCK-8 results showed that the expression levels of IL-1β, TNF-α and IL-6 in the H-Glucose group were significantly increased compared with that of the Control group, while the expression levels of IL-10 and TGF-β were significantly decreased in the H-Glucose group (Figure 3C). It could be concluded that there was inflammation inside HKF

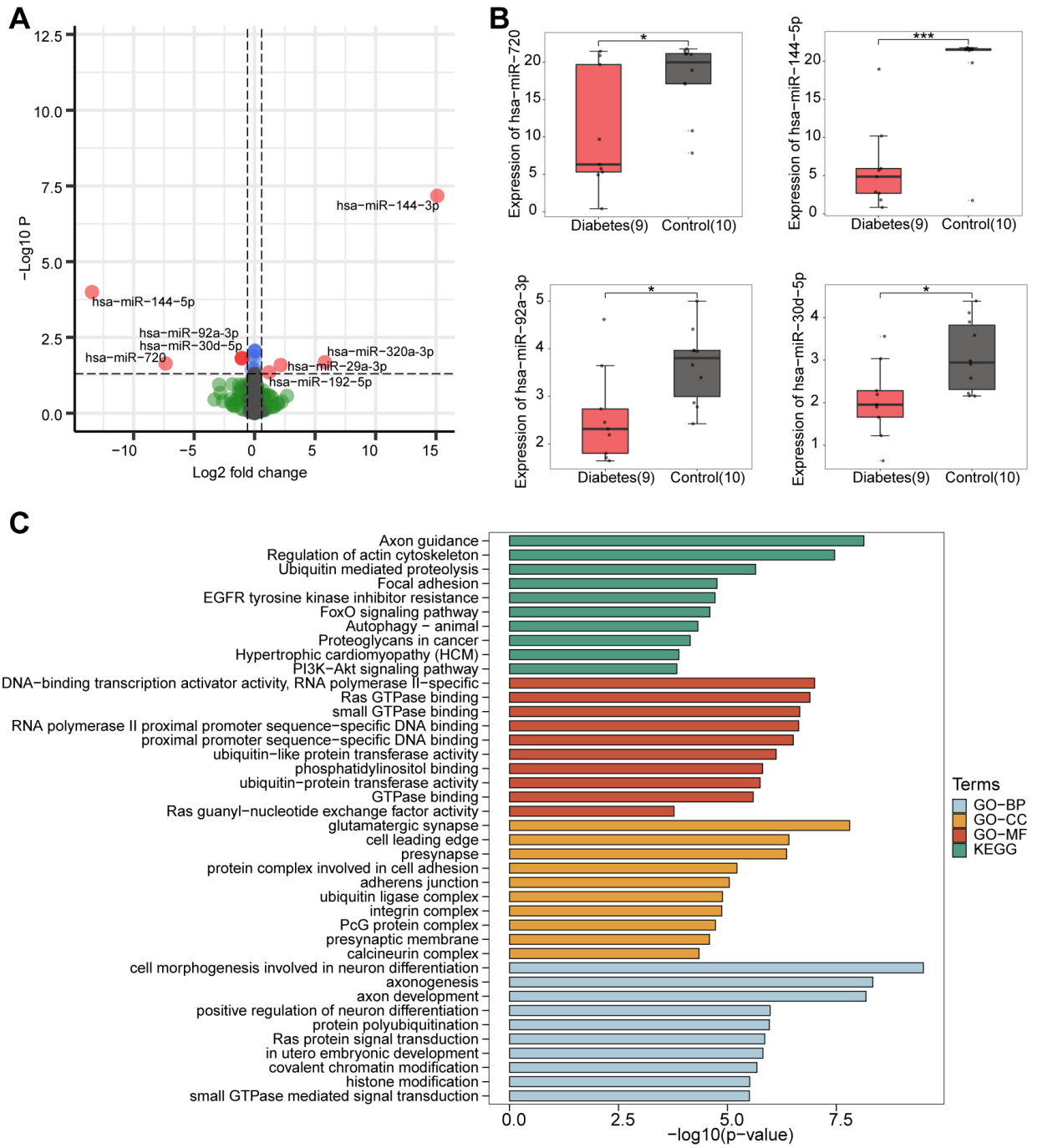

Fig. 1. Differentially expressed miRNAs predicted by bioinformatics in diabetes mellitus. (A) Volcano diagram of significantly differentially expressed miRNAs in hyperglycemia and normal samples. (B) Box diagram of 4 miRNAs with the most significant difference between hyperglycemia and normal samples. (C) GO and KEGG were used to predict the immune pathway mediated by miR-30d-5p.

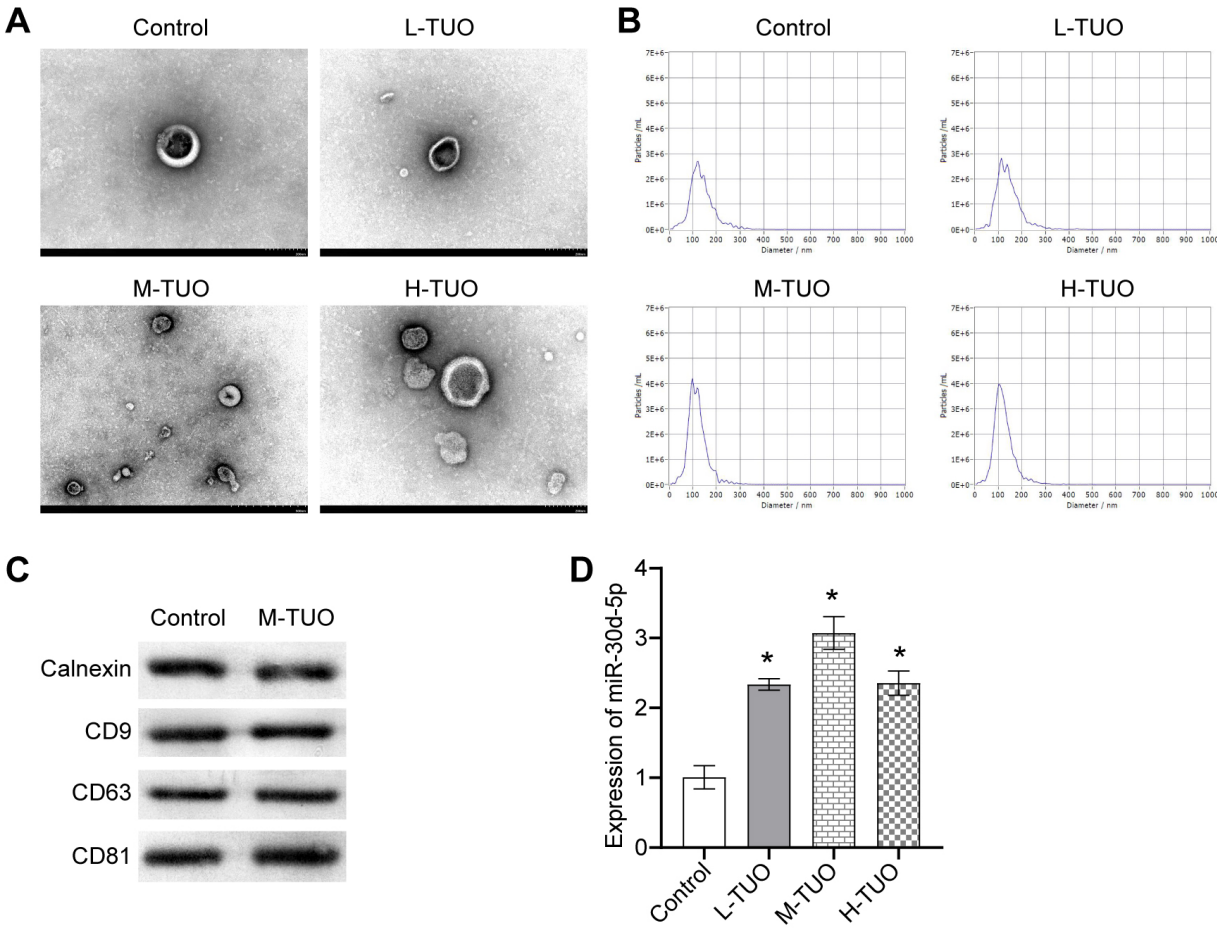

Fig. 2. Effect of tori formula on EPCs-derived exosomes. (A) Observation of EPCs exosomes by TEM. Scale bar = 200 nm in the Control, L-TUO, H-TUO. Scale bar = 500 nm in M-TUO. (B) Measurement of particle size distribution in EPCs exosomes by NTA. (C) Expression of CD81, CD63, CD9 and Calnexin in EPCs-derived exosomes. (D) The expression of miR-30d-5p in each group was detected by qRT-PCR. * $p<0.05$ vs. Control group.

cells in the H-Glucose group due to high glucose environment. In contrast, IL-1 β, TNF-α and IL-6 in the Exosomes group were significantly decreased compared with H-Glucose, while IL-10, TGF-β was significantly increased (Figure 3C), indicating that inflammation response in HKF cells in this group was inhibited. Since exosomes in M-TUO were added to the Exosomes group, it could be speculated that EPCs exosomes could promote the proliferation of HKF cells impaired by high glucose and inhibit inflammation.

EPCs-derived exosomes promoted the polarization of macrophages from M1 to M2 via miR-30d-5p

The expression of miR-30d-5p in macrophages of exo-NC and exo-miR-30d-5p mimic showed that the expression of miR-30d-5p in exo-miR-30d-5p mimic was significantly higher than that in the exo-NC group (Figure 4A), which suggested that miR-30d-5p mimic was successfully transfected into EPCs. ELISA revealed that the inflammatory factor IL-1β, TNF-α and IL-6 in

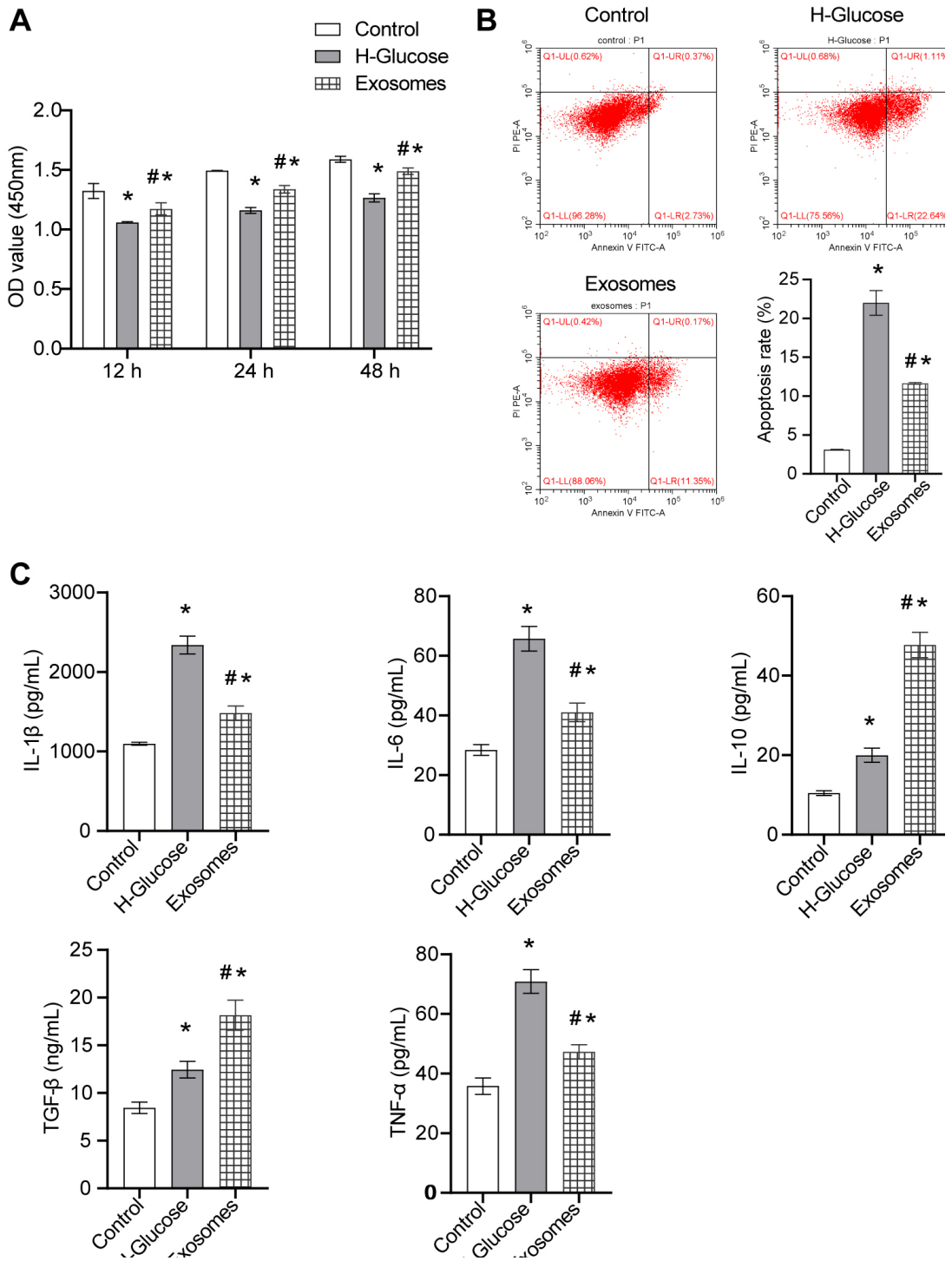

Fig. 3. Effect of EPCs-derived exosomes on HKF. (A) Cell viability of HKF in each group was detected by CCK-8 at 12h, 24h and 48h. (B) Apoptosis was detected by flow cytometry. (C) IL-1β, TNF-α, IL-6, IL-10 and TGF-β in cell supernatant detected by ELISA. * $p<0.05$ vs. Control group, # $p<0.05$ vs. H-Glucose group.

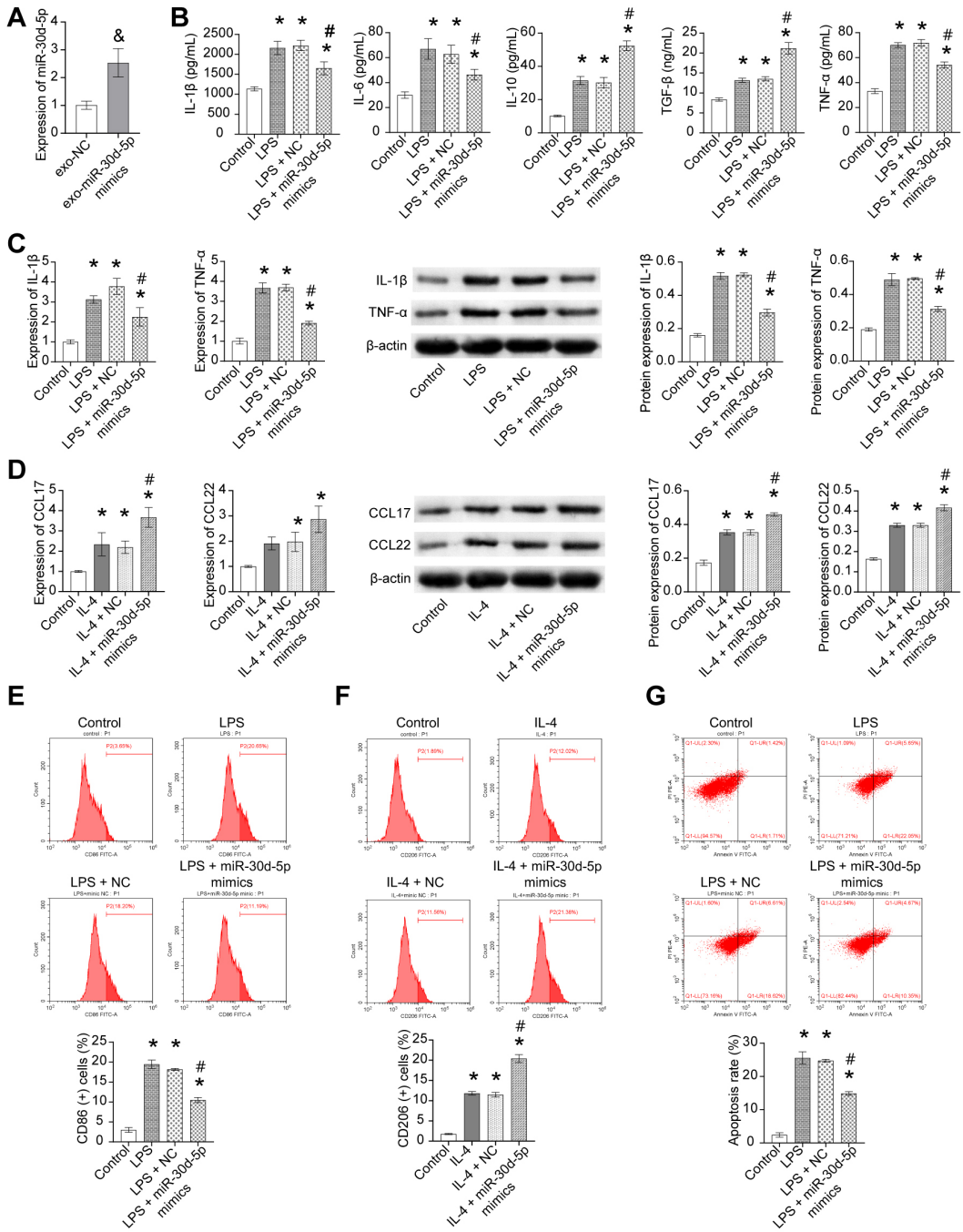

Fig. 4. Effect of miR-30d-5p on the polarization of M1 and M2 macrophages. (A) Expression of miR-30d-5p was detected by qRT-PCR. (B) Expression of IL-1β, TNF-α, IL-6, IL-10 and TGF-β detected by ELISA. (C) Expression of IL-1β and TNF-α in M1- macrophages was detected by qRT-PCR and Western Blot. (D) Expression of CCL22 and CCL17 in M2 macrophages was detected by qRT-PCR and Western Blot. (E) M1 macrophage surface marker CD86 was detected by flow cytometry. (F) M2 macrophage surface marker CD206 was detected by flow cytometry. (G) Cell apoptosis in each group was detected by flow cytometry. & $p<0.05$ vs. exo-NC group,* $p<0.05$ vs. Control group, # $p<0.05$ vs. LPS + NC group or IL-4 + NC group.

LPS+NC groups increased significantly than that of LPS+miR-30d-5p mimic group. In contrast, IL-10 and TGF-β in LPS+NC groups were inhibited compared with those in LPS+miR-30d-5p mimic group (Figure 4B), indicating that miR-30d-5p could inhibit the inflammation response of macrophages. Flow cytometry analysis of M1 macrophages surface antigen CD86, and M2 macrophages surface antigen CD206 showed that the number of CD86-positive cells in LPS+ miR-30d-5p mimic group was lower than that in LPS+NC group. The number of CD206-positive cells in the IL-4+miR-30d-5p mimic group also increased compared with IL-4+NC group (Figure 4E-4F). These results indicated that miR-30d-5p could reduce M1 macrophages, promote the increase of M2 macrophages, and it was reasonable to speculate that miR-30d-5p promoted the polarization of macrophages from M1 to M2. The expressions of IL-1β and TNF-α in LPS+NC group were significantly higher than those in the Control group, while down-regulated in LPS+miR-30d-5p mimic group (Figure 4C).

Similarly, the expressions of CCL17 and CCL22 increased in IL-4 group and IL-4+NC group compared with those in the Control group, and were more significantly up-regulated in the IL-4+miR-30d-5p mimics (Figure 4D). Since IL-1β and TNF-α were specific secretions of M1 macrophages, and CCL17 and CCL22 were signature secretions of M2 macrophages, these results could further verify the polarization of macrophages. Cell apoptosis results demonstrated that the apoptosis level of LPS and LPS+NC groups was significantly increased compared with that of the Control group, and the apoptosis rate of LPS+miR-30d-5p mimic group was significantly decreased compared with the former two groups (Figure 4G). It was indicated that miR-30d-5p mimic could inhibit the apoptosis of macrophages, which also confirmed its inhibitory effect on inflammation in macrophages.

EPCs-derived exosomal miR-30d-5p could promote the polarization of macrophages M1 to M2 to promote the proliferation of high-glucose impaired HKF, inhibit apoptosis and inflammation

QRT-PCR showed that the expression level of miR-30d-5p in the miR-30d-5p mimics group was significantly up-regulated compared with that in the mimics NC group, also down-regulated in miR-30d-5p inhibitor group than that in inhibitor NC group (Figure 5A). These results suggested that miR-30d-5p was successfully transfected in miR-30d-5p mimics and miR-30d-5p inhibitor group. CCK-8 and flow cytometry showed that miR-30d-5p mimics group had a higher cell proliferation ability than mimics NC group. In comparison, miR-30d-5p inhibitor group had a lower level of cell proliferation ability than inhibitor NC group (Figure 5B-5C). It demonstrated that cell proliferation ability was positively correlated with the expression level of miR-30d-5p, suggesting that miR-30d-5p could promote the proliferation of HKF. The expressions of VEGF, FGF21, NRF2 and HO-1 in HKF were detected, and the results also showed the expression levels of these genes were higher in miR-30d-5p mimics group than mimics NC group, while lower in miR-30d-5p inhibitor group than inhibitor NC group (Figure 5D-5E). VEGF was a kind of vascular endothelial growth factor, FGF21 and NRF2 were HKF cell growth factors, HO-1 was heme oxygenase and one of the downstream regulatory factors of NRF2. The expressions of these genes were promoted by miR-30d-5p, suggesting that miR-30d-5p could stimulate the proliferation of EPCs and high-glucose impaired HKF. In addition, the expression levels of MCP-1, IL-1β, TNF-α, IL-6, IL-10 and TGF-β in each group were detected, the results demonstrated the expression of miR-30d-5p was reduced by pro-inflammatory factors such as IL-1β, and enhanced by anti-inflammatory factors such as TGF-β (Figure 5F). It could be speculat-

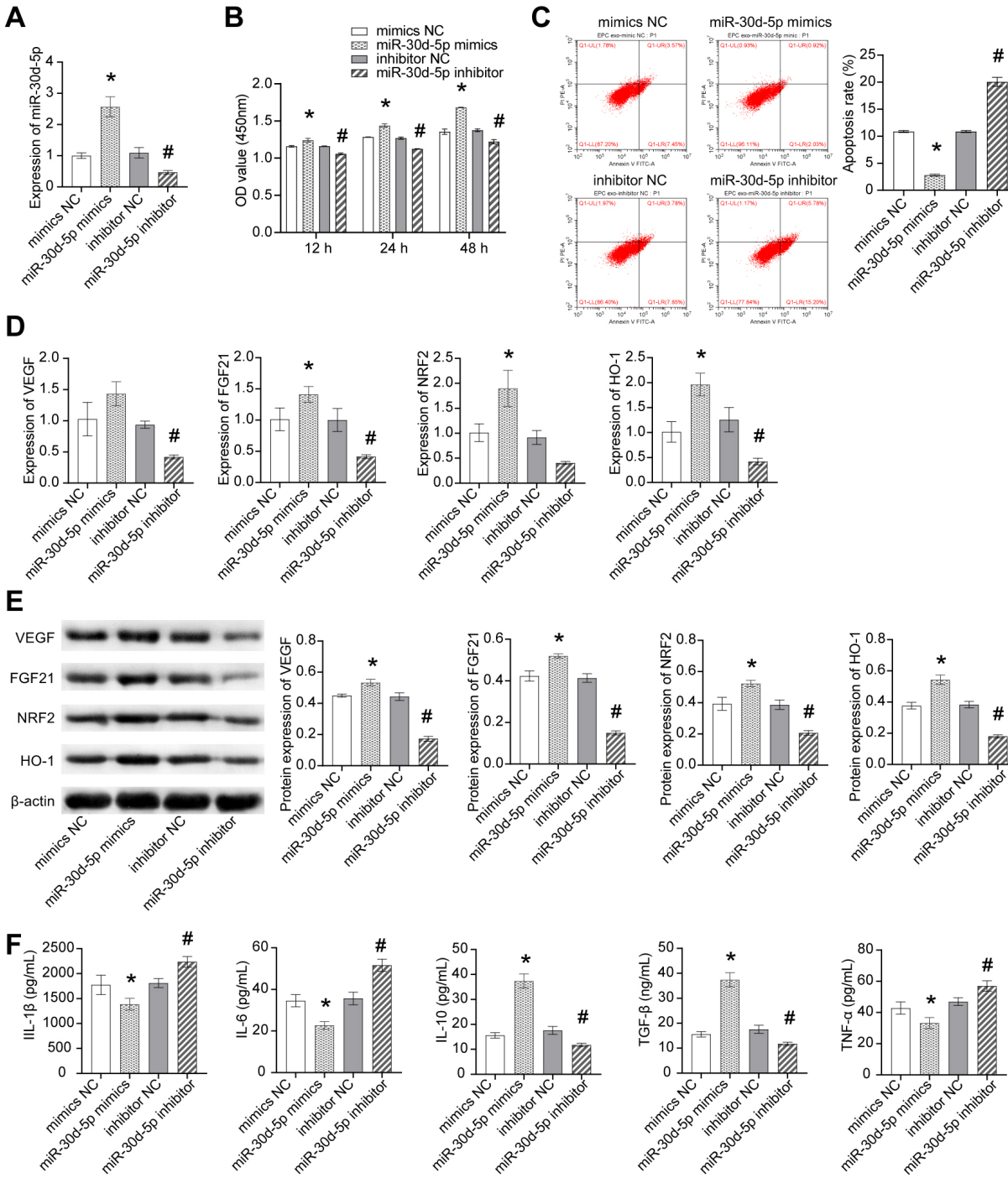

Fig. 5. Effect of miR-30d-5p on high glucose impaired HKF. (A) Expression of miR-30d-5p in HKF in each group was detected by qRT-PCR. (B) The proliferation of HKF at 12 h, 24h and 48h was detected by CCK-8. (C) Apoptosis of cells in each group was detected by flow cytometry. (D-E) Expression of VEGF, FGF21, NRF2 and HO-1 was detected by qRT-PCR and Western Blot. (F) Expression of MCP-1, IL-1β, TNF-α, IL-6, IL-10 and TGF-β in cell supernatant was detected by ELISA. * $p<0.05$ vs. mimics NC group; # $p<0.05$ vs. inhibitor NC group.

ed that miR-30d-5p could inhibit inflammation in high glucose-impaired HKF cells.

Discussion

Diabetes is a chronic disease worldwide. The rapid growth of prevalence and numerous complications have made diabetes a difficult problem threatening social and family health (26). Studies have shown that some exosome-derived miRNAs have a regulatory effect on diabetes (27). In this study, we explored the function and mechanism of EPCS exosome miR-30d-5p in high-glucose impaired HKF.

Many exosome-derived miRNAs are expressed at different levels in diabetic patients (28, 29). We first selected hyperglycemia and normal samples in the GSE21321 dataset, analyzed miRNAs with significantly different expressions between the two groups, finally miR-30d-5p was screened. To explore the relationship between miR-30d-5p and exosome regulation of DM, EPCs were treated with different concentrations of tori formula and exosomes were detected. The results showed that appropriate concentrations of tori formula could promote the secretion of EPCs exosomes, and the expressions level of miR-30d-5p in each group of exosomes was also related to the number of exosomes. These data suggested that miR-30d-5p was a kind of EPCs exosome miRNA with the potential to regulate diabetes.

The occurrence of diabetes is often accompanied by the production of inflammation and cell apoptosis in vivo (30, 31). EPCs exosomes were added to high-glucose impaired HKF, the proliferation and expression of intracellular inflammatory factors were detected. The results showed that EPCs exosomes could alleviate HKF inflammation and apoptosis induced by high glucose. The previous study showed that miR-30d-5p was a miRNA correlated with the content of EPCs exosomes, it was also indicated that miR-30d-5p might alleviate the inflammation and apoptosis of HKF cells. But the mechanism in-depth remained to be further confirmed.

The generation and alleviation of inflammation are closely related to macrophages in the human body (32). M1 macrophages formed by the polarization of macrophages can promote the occurrence of inflammation, which also secrete TNF-α and other inflammatory factors (33). M2 macrophages can secrete high levels of TGF-β and other anti-inflammatory factors, inhibiting the inflammatory effect of M1 macrophages (34). MiR-30d-5p is associated with systemic inflammation (35). To study the regulatory mechanism of miR-30d-5p on cellular inflammation, EPCs exosomes transfected with miR-30d-5p were used to treat LPS/IL-4-induced M1/M2 macrophages. Comparing the expression levels of inflammatory factors such as TNF-α and anti-inflammatory factors such as TGF-β in each group, it was shown that exosomes transfected with miR-30d-5p inhibited the expression of inflammatory factors in M1 macrophages and promoted the expression of anti-inflammatory factors. The detection results of M2 macrophages were also consistent with the previous results, suggesting that miR-30d-5p could inhibit the inflammatory induced by M1 macrophages. M1 and M2 macrophages can be detected by the level of surface signature antigen CD86 and CD206 (36). M1 macrophages can secrete inflammatory factors such as TNF-α (37), and M2 macrophages can secrete CCL17. CCL22 and other chemokines (38), the expression of these indicators can be reflected as the number of M1 and M2 macrophages. The number of M1 macrophages transfected with miR-30d-5p exosomes was reduced and the number of M2 macrophages was increased. Flow cytometry also verified this result, and it was reasonable to infer that miR-30d-5p could promote the polarization of M1 macrophages to M2 macrophages.

Previous studies have shown that EPCs exosomes could inhibit inflammation and apoptosis

in high-glucose impaired HKF cells. MiR-30d-5p could promote the polarization of M1 macrophages to M2 macrophages. It could be assumed that miR-30d-5pwas an important substance in this regulatory pathway. To further confirm the effect of miR-30d-5p, high-glucose impaired HKF treated with EPCs exosome transfected with miR-30d-5p were used, and their proliferation and inflammation levels were detected. Similarly, the apoptosis level and inflammatory response of HKF treated with EPCs exosome expressing a high level of miR-30d-5p were alleviated, which also verified that miR-30d-5p could promote the polarization of M1 macrophages to M2 macrophages. It could also be concluded miR-30d-5p inhibited apoptosis and inflammation of high-glucose impaired HKF, but the specific regulatory mechanism still needs further study.

Conclusion

In this study, EPCs-derived exosomal miR-30d-5p was screened, and its function was explored. It was confirmed that miR-30d-5p could inhibit HKF inflammatory response and apoptosis. Meanwhile, the regulation mechanism of miR-30d-5p was speculated and verified. Finally, it was proposed and verified that miR-30d-5p promoted the proliferation of HKF and inhibited its inflammatory response by promoting the polarization of M1 macrophages to M2 macrophages. This study screened and verified the function of miR-30d-5p in HKF, and proposed a regulatory pathway, providing a new idea for the target and direction of diabetes treatment.

Author Contribution

X Z contributed to conception and design of the study. W X performed the statistical analysis and wrote the first draft of the manuscript. All authors participated in the experiment and approved the final manuscript.

Funding Statement

This study was supported by National Natural Science Foundation of China Youth Science Fund Project (81904217).

Conflicts of Interest

There was no conflict of interest between authors to declare.

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