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Umbilical Cord Mesenchymal Stem Cells-derived Exosomes Deliver Mir-21 to Accelerate Corneal Epithelial Wound Healing Through PTEN/PI3K/Akt pathway

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Research Article

Keywords: Corneal wound healing, Umbilical cord mesenchymal stem cell, Exosomes, miR-21, PTEN

Posted Date: June 8th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-571150/v1

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Abstract

Background

Rapid restoration of corneal epithelium integrity after injury is particularly important for preserving corneal transparency and vision. Mesenchymal stem cells (MSCs) can be taken into account as the promising regenerative therapeutics for improvement of wound healing processes based on the variety of the effective components. The extracellular vesicles form MSCs, especially exosomes, has been considered as important paracrine mediators though transferring microRNAs into recipient cell. This study investigated the mechanism of human umbilical cord MSC-derived exosomes (HUMSC-exosomes) on corneal epithelial wound healing.

Methods

Exosomes extracted from HUMSCs were identified by transmission electron microscopy, nanoparticle tracking analysis, and Western blot. Corneal fluorescein staining and histological staining were evaluated in a corneal mechanical wound model. Changes in HCECs proliferation after HUMSC-exosomes or miR-21 mimic treatment were evaluated by CCK-8 and EdU assays, while migration was assessed by in vitro scratch wound assay. Full-length transcriptome sequencing was performed to identify the differentially expressed genes associated with HUMSC-exosomes treatment, followed by validation via real-time PCR and Western blot.

Results

The exosomes derived from HUMSCs can significantly promote corneal epithelial cells proliferation, migration in vitro and accelerate corneal epithelial wound healing in vivo. Similar effects were obtained after miR-21 transfection, while the beneficial effects of HUMSC-exosomes were partially negated by miR-21 knockdown. Results also show that the benefits are associated with decreased PTEN level and activated the PI3K/Akt signaling pathway in HCECs.

Conclusions

HUMSC-exosomes could accelerate the recovery of corneal epithelial wounds though restraining PTEN by transferring miR-21, and may represent a promising novel therapeutic agent for corneal wound repair.

Introduction

Superficial corneal lesions can heal rapidly and without complication, however, delayed corneal epithelial healing can lead to subsequent corneal infections with further complications, such as corneal scarring, thinning, ulceration and even perforation. According to the World Health Organization, it is estimated that corneal opacities, including corneal ulceration, are the fourth leading cause of blindness worldwide [1]. Although several therapies exist and an increasing number of novel approaches are emerging, treatment of severe corneal epithelial defect can still be quite challenging. Therefore, a topical treatment that aids in

the management and accelerated closure of corneal wounds would help reduce the risk of infections and scarring, and thus improve visual outcomes.

Mesenchymal stem cells (MSCs)-based therapies participated in renovating the structure and function of damaged or diseased tissues[2]. However, poor engraftment and limited differentiation of transplanted MSCs suggest that their beneficial effects might not be associated with their differentiation and direct replenishment of damaged tissue parenchymal cells [3, 4]. Currently, emerging evidence has shown that the therapeutic effect of MSCs mainly relies on paracrine activities [5]. MSCs can release extracellular vesicles (EVs) containing bioactive molecules that affect cellular processes in neighboring cells. Therefore, it may be possible to avoid the limitations and complications of stem cell therapy in the eye by using MSC derived EVs as biomimetic agents to accelerate corneal wound healing [6].

EVs, specifically exosomes, are functional paracrine units of stem cells and have therapeutic effects similar to their parent cells, suggesting that they may provide a promising, cell-free therapeutic options [7–9]. The application of MSC-derived exosomes alone can exert similar functions of MSCs and participate in the regulation of immune response, inflammatory disease and wound healing [10]. The cellular bilayer lipid membrane of exosomes protected proteins, mRNAs, and non-coding RNAs (ncRNAs) from destruction, which can be transferred to recipient cells for cell-to-cell communication [11]. ncRNAs is considered as key post-transcriptional modulators of gene expression and can be transferred in active form via exosomes to regulate the activity of certain cells. Among them, microRNAs (miRNAs) have emerged as the most important modulator [12]. miRNAs are a class of evolutionally conserved, single-stranded ncRNAs, which are either transcribed by RNA polymerase II from independent genes or introns of protein-coding genes [13]. miRNAs are crucial players during normal development, homeostasis, and disease, which participate in almost every biological process such as cell proliferation and survival [14].

MSCs from cord tissues are easily attainable and more primitive than MSCs isolated from adult sources. Previous studies have shown that human umbilical cord mesenchymal stem cell derived exosomes (HUMSC-exosomes) could transfer miRNAs and attenuate cell death and enhance cutaneous wound healing [15, 16].Considering the similar wound healing process between skin and corneal, we studied the functions of HUMSC-exosomes in corneal wound repair. The present study demonstrated the therapeutic effect of HUMSC-exosomes using a corneal mechanical wound model. To investigate the mechanism underlying HUMSC-exosomes-mediated corneal wound repair, we studied the effects of HUMSC-exosomes on human corneal epithelial cells (HCECs) migration and proliferation. Through high-throughput sequencing and bioinformatics analysis, we identified that miR-21 are carried by HUMSC-exosomes as crucial elements contributing to HCECs migration and proliferation by down-regulating PTEN expression.

Materials And Methods

Primary cell culture and Characterization

All experiments were approved by the ethical committee of Harbin Medical University, and informed consent was obtained from healthy donors before the study. The collected umbilical cord Wharton's jelly tissue was cut into small pieces, and then allowed to stick to the bottom of the cell culture plates. Dulbecco's Modified Eagle's Medium (DMEM) Low Glucose with 10% fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin (Gibco, USA) were added to the cells. The dissociated cells were washed with PBS, and stained with antibodies CD90, CD105, CD73, CD34, CD11b, CD19, CD45 and HLA-DR using the BD™ Human MSC Analysis Kit. The FACS analysis was performed using a FACS Calibur™ flow cytometer (BD Biosciences), and the data were analyzed using FlowJo software (BD Biosciences). All cells used in our experiments were from early passages 3 to 5.

To avoid the influence of FBS-derived exosomes on HUMSC-exosomes, HUMSCs used for exosomes extraction were cultured using exosomes-free FBS which were centrifuged at 120,000 g at 4°C for 18 hours using a Beckman Optima L-100 XP ultracentrifuge with a SW 32 Ti rotor.

Isolation and identification of HUMSC-exosomes

HUMSCs supernatants was collected at different times every 24–48 hours and centrifuged at 300 g for 10 minutes to pellet dead cells, and cell debris were removed by centrifuging at 2,000 g for 10 minutes, and then 10,000 g for 30 minutes to eliminate large vesicles, after centrifuging at 120,000 g for 70 minutes, HUMSC-exosomes pellets were washed with PBS and ultracentrifuged at 120,000 g for another 70 minutes. All centrifugation steps were performed at 4°C. The purified exosomes were resuspended in PBS and stored at -80°C.

The concentration of HUMSC-exosomes was determined by BCA protein assay kit, as suggested by the manufacturer (Beyotime Institute of Biotechnology, China). The morphology of HUMSC-exosomes was observed by transmission electron microscopy (TEM) (JEOL JEM-1220, Japan). And the size distribution of HUMSC-exosomes was measured by nanoparticle tracking analysis (NTA, Malvern Zetasizer, England). The membrane protein marker (CD9, CD81, CD63) were analyzed using Western blot.

To obtain the miR-21 knockdown HUMSC-exosomes, we transfected MSCs with miR-21 inhibitors (RIBOBIO, China) or negative control (NC) using Opti-MEM (Gibco, USA) and Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 48 hours of culture incubation, exosomes were isolated from culture supernatants by differential centrifugation as described above.

HCECs culture and transfection

The human corneal epithelial cell line (HCEC, Bnbio, China) were cultured in DMEM High Glucose supplemented with 10% FBS (Gibco, USA). HCECs were seeded into 6-well or 12-well plates the day before treatment. Prior to HUMSC-exosomes or PBS treatment, HCECs were starved in serum-free DMEM for 24 hours at 50% confluence. HCECs were transfected with miR-21 mimics or miR-21 inhibitors and corresponding NC, pCDNA3.1-HA-PTEN and empty vector plasmid as indicated.

Exosome uptake assay

Purified HUMSC-exosomes were stained with Dil (Beyotime Institute of Biotechnology, China) according to the manufacturer's instructions and were then washed and resuspended in serum-free medium. CFSE (Invitrogen, USA) labeled HCECs were seeded into glass bottom dishes (Cellvis, USA) for single layer and then co-cultured with Dil-labeled HUMSC-exosomes for 2 hours. After washed with PBS and fixed in 4% paraformaldehyde, cell nuclei were stained with DAPI (Beyotime Institute of Biotechnology, China). Images were taken under confocal microscope (Zeiss LSM 710, Germany) and analyzed with supplementary software.

For TEM observation, HCECs were co-cultured with exosomes for 2 hours and then fixed with 2.5% glutaraldehyde and postfixed with 3% osmium tetroxide (OsO4) for 2 hours. The specimen was dehydrated in a graded series of ethanol, embedded in Epon resin and then imaged with TEM at 100 kV (Hitachi H-7650, Japan).

In vitro Wound healing assay

HCECs were planted into six-well plates and grown to confluence. The monolayer was scratched using a 200μl pipette tip and washed with serum-free medium to remove detached cells. Then, the cells were kept in co-culture with HUMSC-exosomes or not. At different times, images of wound scratch were taken under a microscope. The scratch closure was analyzed by ImageJ software. The percentage of wound closure was calculated as follows: migration area (%) = $(A_0 - A_n)/A_0 \times 100$, where A_0 represents the initial wound area, and A_n represents the wound area at the time of measurement.

In vitro Cell proliferation assay

HCECs proliferation was measured using the cell counting kit-8 (CCK-8, Sigma, USA) according to the manufacturer’s protocol. The optical density (OD) at 450 nm was measured with averages from three replicates using a microplate reader (BioTek Instruments, USA).

In vitro EdU proliferation assay

Cell proliferation was also assessed using EdU Cell Proliferation Assay kit (RiboBio, China) according to the manufacturer’s protocol. Briefly, after treatment, HCECs were exposed to 50 μM 5-ethynyl-2'-deoxyuridine (EdU, RiboBio) for 2 h at 37°C, and then the cells were fixed in 4% paraformaldehyde. After permeabilization with 0.5% Triton-X100, the cells were reacted with 1× Apollo reaction cocktail for 30 minutes. Subsequently, the DNA contents of the cells were stained with Hoechst33342 for 30 minutes. Finally, the proportion of the cells incorporating EdU was determined with fluorescence microscopy (OLYMPUS, IX51).

Western blot

HUMSC-exosomes and HCECs were lysed in lysis buffer containing a complete protease inhibitor tablet (Roche, Swiss). Proteins was separated by electrophoresis after loading onto polyacrylamide gel, and then transferred to the PVDF that was incubated with primary antibodies against phospho-Akt (4060s, CST), phospho-PI3K (4228s, CST), PTEN (9188, CST), cyclin D1 (ab134175, Abcam), CD9 (ab92726,

Abcam), CD61 (ab59479, Abcam), CD81 (00679767, Invitrogen) overnight at 4°C after blocking with 5% nonfat milk, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody. Proteins were detected with a Western blot analysis system (Universal Hood II, Bio-Rad).

PCR

Total RNA of cells was extracted using TRIzol kit (Invitrogen, USA). RT-qPCR was carried out using the SYBR® Premix Ex TaqTM kit (Takara Bio, Japan) according to the manufacturer's instructions. The thermocycling conditions (Bio-Rad, CFX96) used were as follows: 95°C for 3 minutes; followed by 40 cycles of 95°C for 5 seconds, 60°C for 30 seconds and 72°C for 30 seconds, followed by a final extension of 72°C for 5 minutes. Relative expression of these genes was calculated by the $2^{-\Delta\Delta Ct}$ method.

Bioinformatics analysis

HUMSC-exosomes microRNA expression microarray GSE69909 were downloaded from GEO database. Target Scan, mirBase and miRDB were used to predict the target genes of miRNAs enriched in exosomes. All the predicted targets have target prediction scores ≥ 80 were subjected to gene ontology (GO) analysis to investigate the underlying mechanism of the potential HUMSC-exosomes miRNA and the target mRNAs during corneal re-epithelialization.

Full-length transcriptome sequencing

HCECs (2.5×10^6 cells) were treated with 40μg/ml HUMSC- exosomes for 48 hours, same volume of PBS was added as control with three biological replicates. Total RNA was isolated using the TRIzol reagent according to the manufacturer's instructions. 1ug total RNA was prepared for cDNA libraries using cDNA-PCR Sequencing Kit (SQK-PCS109) protocol provided by Oxford Nanopore Technologies (ONT). Briefly, the template switching activity of reverse transcriptase enrich for full-length cDNAs and add defined PCR adapters directly to both ends of the first-strand cDNA. And following cDNA PCR for 14 circles with LongAmp Tag (NEB). The PCR products were then subjected to ONT adaptor ligation using T4 DNA ligase (NEB). Agencourt XP beads was used for DNA purification according to ONT protocol. The final cDNA libraries were added to FLO-MIN109 flowcells and run on PromethION platform at Biomarker Technology Company (Beijing, China). KOBAS software was used to test the statistical enrichment of differential expression transcripts in KEGG pathways.

Corneal mechanical wound model and HUMSC-exosomes treatment

Weight from 160–180g male Sprague-Dawley rats were purchased from the animal experiment center of the Second Affiliated Hospital of Harbin Medical University. All animals were kept adaptively for additional a week and randomly divided into two groups: the PBS group and the exosomes treatment group. The rats were anesthetized with intraperitoneal injection and applied topically 0.5% proparacaine, a 2-mm area of the central epithelium was demarcated and removed by an AlgerBrush II (The Alger

Company, Lago Vista, TX, USA) as previously described [17]. A unilateral corneal injury was created. Protocols were approved by the Harbin Medical University Animal Care and Use Committee guideline.

The wounded corneas were then treated topically with HUMSC-exosomes or the PBS every 6 hours. Wound residual area was monitored every 12 hours using fluorescein staining and photographed using a camera equipped Nikon FS-2 slit lamp biomicroscope. The percentages of residual defect were analyzed by ImageJ software.

Histological analysis

The eyes were enucleated and post-fixed with 4% paraformaldehyde within 10 minutes after euthanasia. 4μm paraffin-embedded sections stained with hematoxylin and eosin (H&E) were used to observe the corneal structure and degree of corneal re-epithelialization. The sections were photographed under light microscope (Olympus, Japan).

Statistical analysis

All statistical analyses were performed using Prism software. Data are summarized as mean ± standard deviation (SD). Student *t*-test was used to determine statistically significant differences between samples. When multiple comparison analyses were required, statistical significance was evaluated by one-way ANOVA. All *P*-values < 0.05 were considered statistically significant.

Results

1. Identification of HUMSCs and HUMSC-exosomes

Results of flow cytometry analysis confirmed the presence of positive expressions of typical MSC makers CD105, CD90, CD73, while the surface markers of hematopoietic cells such as CD34, CD11b, CD19, CD45 and HLA-DR were fairly weak to detect compared with the isotype control (Fig. 1a). In addition, according to inverted microscopic observation, the morphology of the cells was regular long spindle with directional arrangement, and presented a typical spindle shape, which grew as whirlpool or cluster (Fig. 1b).

The classical structure of the isolated exosome, including “rim of a cup” and double-layer membrane morphology, were observed by TEM (Fig. 1c). NTA results demonstrated that the diameters of the particles were around 50–150 nm (Fig. 1d). The identity of these particles was further confirmed as exosomes by Western blot, which showed the presence of widely expressed exosomal markers, including CD9, CD63, CD81 (Fig. 1e). Therefore, results above confirmed that the EVs we extracted were indeed exosomes.

Morphological observation and identification of HUMSCs and HUMSC-exosomes. **a** Flow cytometry analysis of surface markers in HUMSCs. **b** Light morphology image of HUMSCs. **c** Morphology of HUMSC-exosomes under TEM. Scale bar, 100 nm. **d** Peak size of HUMSC-exosomes was around 80 nm

as showed by NTA. **e** HUMSC-exosomes were positive for CD9, CD81 and CD63 as indicated by Western blot.

2. Topical application of HUMSC-exosomes accelerates corneal wound healing in a rat model

To investigate the influences of HUMSC-exosomes on mechanical corneal epithelium defect model, 5 μL of 1 μg/μL exosome suspensions or PBS were topically dropped on defect eye four times a day. The change of corneal defect area was monitored using the corneal fluorescein staining every 12 hours. HUMSC-exosomes treatment significantly decreased the percentages of residual defect compared to the PBS-treated group at 48 hours. (Fig. 2a-b). The injured corneas treated with HUMSC-exosomes regained more regular arrangement and compact structure than those treated with PBS through assessing corneal tissues microstructure by H&E staining. In addition, corneal epithelium detachment and inflammatory cells infiltration occurred in the PBS group (Fig. 2c).

The effect of HUMSC-exosomes on corneal epithelial wound healing in vivo. **a, b** Fluorescein-stained images of defect corneas, before and after treatment with HUMSC-exosomes or PBS. Wounds treated with HUMSC-exosomes had healed significantly more than control. **c** H&E staining showed the histologic appearance of the cornea in exosome-group mice and PBS-group mice at 72 h. Data are expressed as the means ± SD. $*P < 0.05$.

3. HUMSC-exosomes promote the proliferation and migration of HCECs in vitro

To demonstrate the uptake of exosomes, CFSE-labelled HCECs were co-cultured with Dil-labelled exosomes and then visualized with laser scanning confocal microscope. Localization results showed that exosomes derived from HUMSCs had been taken up by HCECs with the dye distributing within in the cell (Fig. 3a). In addition, the fusion process was also observed by TEM (Fig. 3b).

In order to evaluate whether HUMSC-exosomes stimulates HCECs migration, the effect on wound closure rates were investigated. The disparity of the remaining area during scratch wound assays confirmed the promigratory effects of HUMSC-exosomes with a dose-related trend after 18 hours incubation (Fig. 3c-d). Considering corneal healing is a dynamic interwoven process composed of cell proliferation, migration and adhesion, and the proliferation ability is the basis. We further investigated whether HUMSC-exosomes could enhance the proliferation-promoting behavior of HCECs in vitro. The CCK-8 assay showed that the proliferation of HCECs after incubating with exosomes was significantly improved in a dose-dependent manner (Fig. 3e). And the EdU assays for visualization of proliferating cells also demonstrated that HUMSC-exosomes treatment increased the percentage of proliferating cells compared to controls (Fig. 3f-g).

The effect of HUMSC-exosomes on HCECs proliferation and migration in vitro. **a** Fluorescence images of CFSE-labelled HCECs (green) incubated with Dil-labelled HUMSC-exosomes (red). Nuclei were stained

with DAPI (blue). **b** TEM of HCECs incubated with HUMSC-exosomes. **c, d** Representative images from in vitro scratch wound healing assays demonstrating that cell migrate into the cell-free region is significantly accelerated in the presence of HUMSC-exosomes when compared to controls. **e** CCK-8 assay showed increased proliferation of HCECs incubated with HUMSC-exosomes after 48 hours. **f, g** The proliferating HCECs was detected by EdU incorporation. The cells were treated with HUMSC-exosomes or blank control. Blue: nuclear staining (Hoechst33342); Red: EdU staining. **h, i** The expression of phospho-PI3K, phospho-Akt and cyclin D1 were detected through Western blot. Data are expressed as the means ± SD. *P< 0.05, **P< 0.01, and ***P< 0.001.

4. HUMSC-exosomes promote HCECs proliferation and migration though PI3K/Akt pathway

To further investigate the potential mechanism of HUMSC-exosomes regulated proliferation and migration in HCECs, full-length transcriptome sequencing was used to detect the mRNA expression levels of related genes. 240 differentially expressed genes (DEG) were identified (Fold Change ≥ 2 and P value < 0.05), including 104 up-regulated DEGs and 136 down-regulated DEGs (Fig. 4a). Then, we interpreted the potential biological functions of DEGs from the gene function and signaling pathway through KEGG enrichment analysis, and revealed that the PI3K/Akt signaling pathways had a significant difference between before and after HUMSC-exosome treated HCECs (Fig. 4b).

Previous studies demonstrated that PI3K/Akt pathway involved deeply in the modulation of the process of corneal epithelial wound healing [18].Therefore, the PI3K/Akt signaling pathways involved in HCECs proliferation and migration process after HUMSC-exosomes treatment were explored. The activation of the PI3K/Akt pathway in HCECs following HUMSC-exosome stimulation was verified by treating the cells with HUMSC-exosomes or PBS for 24 hours and assessing the phosphorylated protein levels (Fig. 3h). In addition, since the PI3K/Akt pathway is a strong activator of cyclin D1 which plays a critical role in regulating cell cycle from G1 phase to S phase [19], we reasoned that exosomes may exert its effects on proliferation through modulating the cell phase transition. As expected, the protein levels of cyclin D1 were also increased after HUMSC-exosomes treatment. (Fig. 3h).

Transcriptome and pathway analysis of HUMSC-exosomes treatment. **a** Volcano Plot of DEGs between HUMSC-exosomes treated and untreated HCECs. Dots in green stands for down-regulated DEGs and red dots mean up-regulated DEGs, black dots are non-significant DEGs. **b** The KEGG annotation results of the DEGs were classified according to the pathway types in KEGG. DEG, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes

5. HUMSC-exosomes promote HCECs proliferation and migration through miR-21

Exosomes regulate a large number of physiological activities via exosomal miRNAs [20]. As miRNAs are abundant in HUMSC-derived exosomes, we hypothesized that HUMSC-exosomes promote the healing of corneal epithelial defect mainly through miRNAs. The downloaded dataset was used to determine the

content of various miRNAs in HUMSC-exosomes [21]. Among the several miRNAs selectively enriched in HUMSC-exosomes, we focused on the most abundant one, miR-21(Fig. 5a). The downstream targets were predicted by Target Scan, mirBase and miRDB database, then imputed DAVID online to conduct GO analysis. The results showed that miR-21 was involved in the regulation of various molecular function, containing calcium ion binding, peptidase inhibitor activity, growth factor activity etc. (Fig. 5b). Among them, the phosphatase activity may involve in the regulation of PI3K/Akt.

In order to assess whether the exosome-mediated miR-21 transfer plays a role in HCECs proliferation and migration, a subsequent knockdown experiment was conducted. HUMSCs were transfected with miR-21 inhibitors (at final concentration of 100 nM) or NC, and the culture supernatants were collected subsequently for isolating the exosomes. Then, HCECs were incubated with the same concentration of miR-21 contained or miR-21 knockdown HUMSC-exosomes for migration and CCK-8 analysis. Results showed that the up-regulation of migration (Fig. 6a, d), as well as proliferation (Fig. 6e) induced by HUMSC-exosomes were partially negated by miR-21 knockdown.

To further study the potential involvement of miR-21, HCECs were transiently transfected with miR-21 mimics or NC. Proliferation of HCECs following transfection with miR-21 mimics or NC was assessed using CCK-8 and EdU assay. As shown in Fig. 7c, transient transfection of miRNA mimic is efficient. miR-21 mimics transfection significantly promoted the proliferation of HCECs compared with the NC group (Fig. 6b, f, g). In addition, the ability of HCECs transfected with miR-21 mimics to regain monolayer integrity was raise compared with NC-transfected cells (Fig. 6c, h).

miR-21 involved in the process of HUMSC-exosomes promote cell proliferation and migration. **a, d** HCECs were treated with miR-21KD HUMSC-exosomes or miR-21 contained HUMSC-exosomes for 18h. The scratch assay showed the healing of the miR-21KD HUMSC-exosomes treated group was slower than the miR-21 contained HUMSC-exosomes treated group. **b, f** The proliferation of HCECs was detected by EdU incorporation after transfected with miR-21 mimics (at final concentration of 50 nM). Blue: nuclear staining (Hoechst33342); Red: EdU staining. **e** The CCK-8 assay showed the proliferation of the miR-21 KD HUMSC-exosomes treated group was lower than the miR-21 contained HUMSC-exosomes treated group after 18 hours. **c, h** The scratch assay showed significantly faster wound closure in HCECs incubated with miR-21 mimics than NC after 18 hours. **g** The CCK-8 assay showed the proliferation of the miR-21 mimics group was higher than control group after 48 hours. Data are expressed as the means ± SD. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. KD, knockdown

6. miR-21 regulates cell proliferation and migration by targeting PTEN

Unsurprisingly, we found that the effects of miR-21 on the PI3K/Akt phosphorylation and cyclin D1 protein expression were stimulative (Fig. 7g). miRNAs can exert their functions by interacting with the 3' untranslated region (3' UTR) or protein coding sequence of target mRNAs. According to the miRbase database, PTEN might be the potential downstream of miR-21 (Fig. 7a). To confirm whether PTEN is a target of miR-21 in HCECs, we further measured the expression of PTEN in HCECs transfected

independently with miR-21 mimics or inhibitors and their corresponding NC to verify the interaction between the miR-21 and PTEN by qRT–PCR and Western blot. Once transfected with miR-21 mimics, the protein levels of PTEN were significantly reduced in HCECs (Fig. 7f), and the difference was also detected in transcription level (Fig. 7d). Meanwhile, the inhibition of miR-21 resulted in opposite effect on PTEN expression (Fig. 7f). These results suggested that miR-21 regulate PTEN within HCECs via post-transcriptional modify. Reduced mRNA and protein expression levels of PTEN were identified within HCECs after treated with HUMSC-exosomes (Fig. 7b, e), whereas PI3K/Akt phosphorylation and cyclin D1 expression were increased (Fig. 3h). PTEN down-regulated the PI3K/Akt pathway, which was important for proliferation and migration (Fig. 7h).

Taken together, our data indicate that exosomal miR-21 promotes proliferation by activating PTEN/PI3K/Akt signaling pathway, which might play a critical role to enhance corneal epithelial wound healing.

Exosomal miR-21 regulate HCECs proliferation and migration by activating PI3K/Akt pathway through targeting PTEN. **a** The binding site between miR-21 and PTEN mRNA. **b, e, f** HUMSC-exosomes treatment decreased the RNA and protein levels of PTEN in HCECs. **c** The expression level of miR-21 in HCECs. **d, g, h** The expression of PTEN changed with miR-21 variation. **i, j** The expression level of PTEN, phospho-PI3K, phospho-Akt and cyclin D1 after transfected with miR-21 mimics were detected by Western blot. **k, l** The expression of phospho-PI3K, phospho-Akt and cyclin D1 after overexpression of PTEN. Data are expressed as the means ± SD. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

7. Schematic diagram

Schematic diagram describes the mechanism of HUMSC-exosomes in corneal epithelial defect.

We first determined the positive effects of exosomes derived from HUMSCs in promoting corneal epithelial wound healing, then determined the main molecules PI3K/Akt in the wound healing process and the fact that miR-21 was the most abundantly contained in exosomes through bioinformatics analysis. Finally, we anchored PTEN as the downstream target of miR-21, which was the key link between miR-21 and related protein, and determined that PTEN/PI3K/Akt were involved in cell proliferation and migration.

Discussion

Corneal epithelial damage is one of the most common ocular disorders, and novel treatments are needed to improve clinical outcomes for this type of disease. The current study demonstrated the beneficial effect of HUMSC-exosomes on corneal injury. To elucidate the potential mechanism associated with this activity, our in vitro results revealed that HUMSC-exosomes promotes HCECs proliferation and migration via repression of PTEN expression and downstream effects involving the phosphorylation of Akt and PI3K, and the expression of Cyclin D1. Moreover, exosomal miR-21 as an important regulator also showed the effect in promoting HCECs proliferation and migration by targeting PTEN. Our results suggested that

HUMSC-exosomes may be an exceptionally meaningful and promising approach for the healing of corneal defects.

Researches have shown that MSC-exosomes can play a major role in promoting the repair of damaged tissues [22]. Moreover, MSC-exosomes have many advantages over MSCs, such as less safety concerns [23], long-term preservation and easy transportation [24], lower immunogenicity [25], capacity to cross biological barriers [26]. Previous studies have shown that exosomes from corneal MSCs can reduce scar formation and increase the transparency of corneal healing [17]. Exosomes from placental MSCs can reduce the inflammatory response during corneal alkali burn and promote the restoration of normal corneal structure [27]. In present study, for the first time, we proved that HUMSC-exosomes could promote the repair of corneal epithelium integrity and accelerate the healing process of corneal injury both in vitro and in vivo.

Since miRNAs was first identified by Lee RC, new miRNAs are still being discovered with the development of high-throughput sequencing technologies and computational and bioinformatics prediction methods [28]. Increasing evidences indicated that exosomal miRNAs can prevent target mRNA from translating into protein as posttranscriptional regulation [21]. In most cases, miRNAs interact with the 3' UTR of target mRNAs in a complementary manner to suppress protein translation and then regulate cell proliferation, differentiation, development and senescence [29, 30]. Acting as the crucial mediators of MSC-exosomes, miRNAs can provide sustained therapeutic effect and fundamental alterations of the local microenvironment, making it an ideal therapeutic biomolecule [31]. Many researches have validated the role of miRNAs in exosomes in various types of cells [32, 33]. In order to further explore how HUMSC-exosomes affects the corneal epithelial cells, we consulted GEO dataset and combined with bioinformatic analysis methods to analyze the content composition of exosomal miRNAs, and transcriptome sequencing was performed to identify the DEGs in HUMSC-exosomes treated HCECs compared to untreated condition. We found that exosomes derived from HUMSCs were rich in miR-21, which might act as the physiological and pathological regulatory factor. In our study, the exosomes extracted from miR-21 KD HUMSCs weaken the effect on HCECs proliferation and migration compared with those extracted form miR-21 contained HUMSCs, implicating the function of HUMSC-exosomes partly depends on miR-21. miR-21 overexpression has the similar effect on promoting proliferation and migration of corneal epithelial cells. These results showed that miR-21 has a fundamental function on corneal epithelial cell amplification.

PI3K/Akt pathway is a signal transduction pathway closely related to cell growth and proliferation, and plays an important mediating role in proliferation, differentiation and apoptosis of normal cells. The signal protein activity was increased in the tissue cells with strong proliferation ability [34].Studies have shown that the activation of PI3K and Akt can trigger and accelerate the transformation and proliferation of skin epithelial cells, while the use of inhibitors can inhibit the proliferation of cancer cells and improve the level of programmed cell death [35, 36]. Once the PI3K/Akt Signaling pathway was suppressed, corneal epithelial migration was delayed [37–39]. These observations from various experiments suggest that PI3K/Akt signaling may have the stimulatory effect in the maintenance of the corneal epithelium

integrity. In our experiment, PI3K/Akt pathways were activated in HCECs proliferation and migration promoted by HUMSC-exosomes. miR-21 could weaken the expression level of PTEN, and increase PI3K/Akt signaling activation in HCECs.

The downstream of miR-21 has been verified based on the starBase database prediction, dual-luciferase reporter gene assay and evidences form other researches [40–42]. PTEN was the potential effector, which belongs to tumor suppressor gene and inhibits the phosphorylation level of key proteins in various signaling pathways to play a negative function by promoting cell apoptosis and cell cycle arrest, and regulating cell migration and other links [43]. Recent studies have shown that PTEN is involved in the pathological mechanism of myocardial injury and neurocognition, also in regulating corneal epithelial defects [39, 44–46]. In addition, PTEN remains the main negative regulator of PI3K/Akt signaling through its phosphoinositide phosphatase activity [47].

To confirm the relationship among miR-21, PTEN, PI3K and Akt, we transferred miR-21mimics into HCECs, and found that miR-21 overexpression could down-regulate the expression level of PTEN, and this down-regulation further induced the up-regulation of phospho-Akt and phospho-PI3K. These results demonstrated that miR-21 promoted HCECs proliferation and migration by regulating PI3K and Akt via PTEN. Cyclin D1, is a cyclin protein involved in cell cycle transition. It binds to cyclin-dependent kinases and is influenced by phosphorylation to promote cells to enter S phase [48]. We showed that after the overexpression of miR-21, the level of cyclin D1 protein was up-regulated. PTEN could also regulate the cell cycle by down-regulating the expression of cyclin D1 through its protein phosphatase activity [49]. We found that miR-21 derived from HUMSC-exosomes can promote proliferation through PTEN/PI3K/Akt pathway.

In this study, we have shown that topical application of HUMSC-exosomes facilitated corneal epithelial wound healing. Exosomes are nano-sized vesicles which could be delivered using a needle as small as possible, and their biological activity would not be affected by the increased inner pressure of the needle. We proposed that HUMSC-exosomes can not only be used as a local drug to promote corneal epithelial defects, but also can be injected for more intraocular diseases that cannot be treated locally, thus serving as a putative therapeutic agent. Although we substantiated that miR-21 in HUMSC derived-exosomes mediated the effect of proliferation and migration in HCECs, there still remains unclear whether other exosomal cargoes (protein, DNA, liquid) function as similar roles awaits further investigations. However, our study proved that the administration of HUMSC-exosomes eye drops is a promising strategy for the treatment of corneal epithelial defect, which serves as a foundation for the development of more effective strategy in corneal wound healing.

Conclusion

In conclusion, this study firstly revealed the function of HUMSC-exosomes in promoting corneal epithelial cell proliferation and migration via up-regulating the PI3K/Akt signaling pathway though restraining

PTEN by transferring miR-21, leading to accelerated corneal wound repair and regeneration. Our results offer a novel therapeutic agent for the treatment of a corneal wound as a cell-free therapy.

Abbreviations

MSCs: Mesenchymal stem cells

HUMSC-exosomes: human umbilical cord MSC-derived exosomes

HCECs: human corneal epithelial cells

EVs: extracellular vesicles

ncRNAs: non-coding RNAs

miRNAs: microRNAs

PTEN: Phosphate and tension homology deleted on chromosome

PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase

Akt: protein kinase B

DMEM: Dulbecco's Modified Eagle's Medium

FBS: Low Glucose with 10% fetal bovine serum

CCK-8: Cell Counting Kit-8

TEM: transmission electron microscopy

NTA: nanoparticle tracking analysis

NC: negative control

OD: optical density

EdU: 5-ethynyl-2'-deoxyuridine

SD: standard deviation

GO: gene ontology

DEG: differentially expressed genes

Declarations

Acknowledgements

Not applicable.

Author Contributions

X Li and X Liu participated in the study design and drafted the manuscript. Z Liu, X Ye, Y Li, D Yang, Y Teng, C Shi, S Wang, F Cao and Q Kong contributed to cellular and molecular experiments. Y Zhang, Y Yu, X Li, X Jin and Y Liu contributed to the animal experiments. Sen Qi contributed to the histopathological experiments. ZW and HZ participated in the study design, gave financial support, and proof-read the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the National Natural Science Foundation of China (Grant No. U20A20363 81970776), the Natural Science Foundation of Heilongjiang Province, China (Grant No. LH2020H039), Heilongjiang Province Postdoctoral Research Start-up Fund (LBH-Q19138).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

The animal protocols used in this study and the research proposal for use of human samples were approved by The Ethics Committee of First Affiliated Hospital of Harbin Medical University and in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

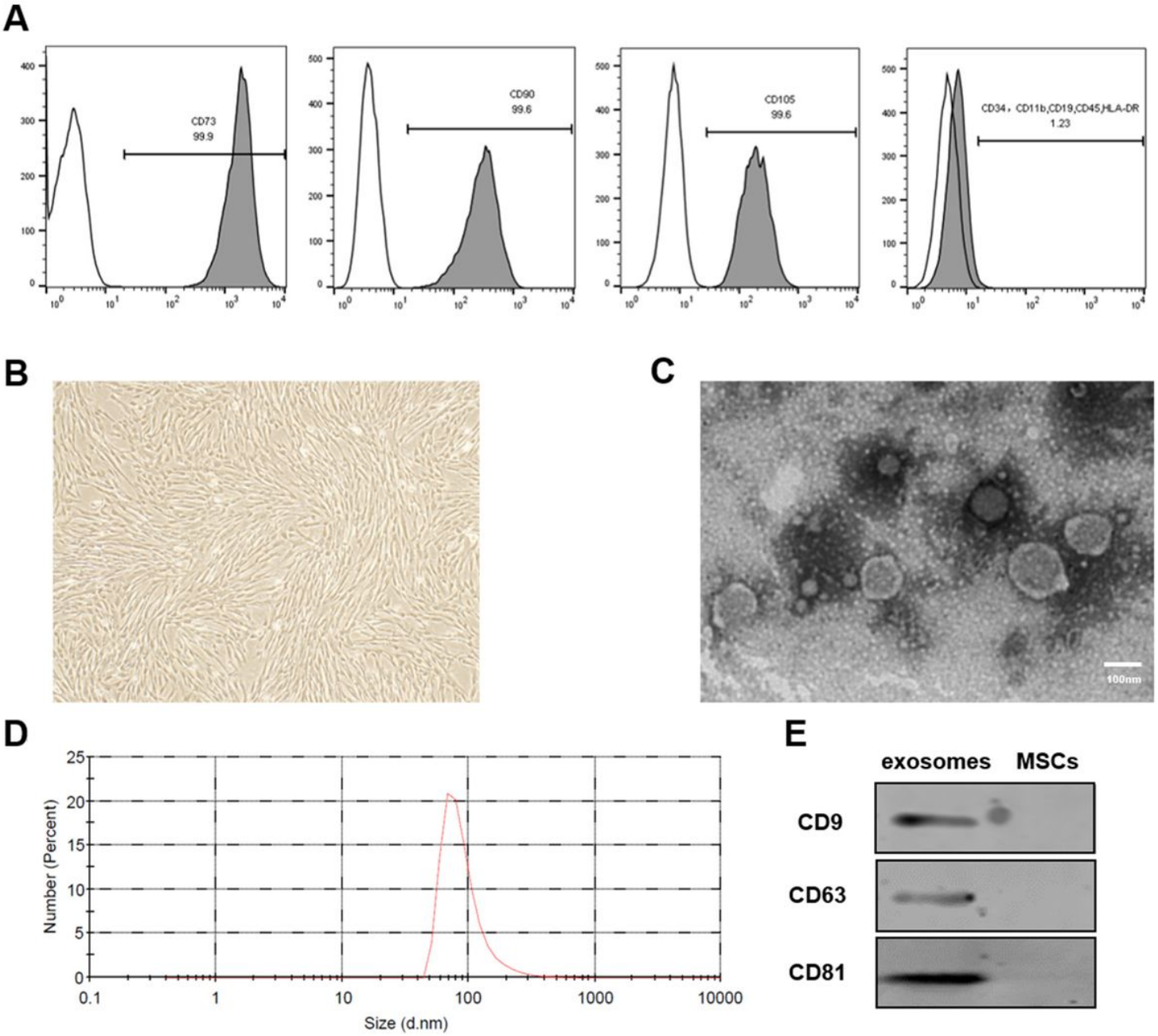

Figure 1

Morphological observation and identification of HUMSCs and HUMSC-exosomes. a Flow cytometry analysis of surface markers in HUMSCs. b Light morphology image of HUMSCs. c Morphology of HUMSC-exosomes under TEM. Scale bar, 100 nm. d Peak size of HUMSC-exosomes was around 80 nm as showed by NTA. e HUMSC-exosomes were positive for CD9, CD81 and CD63 as indicated by Western blot.

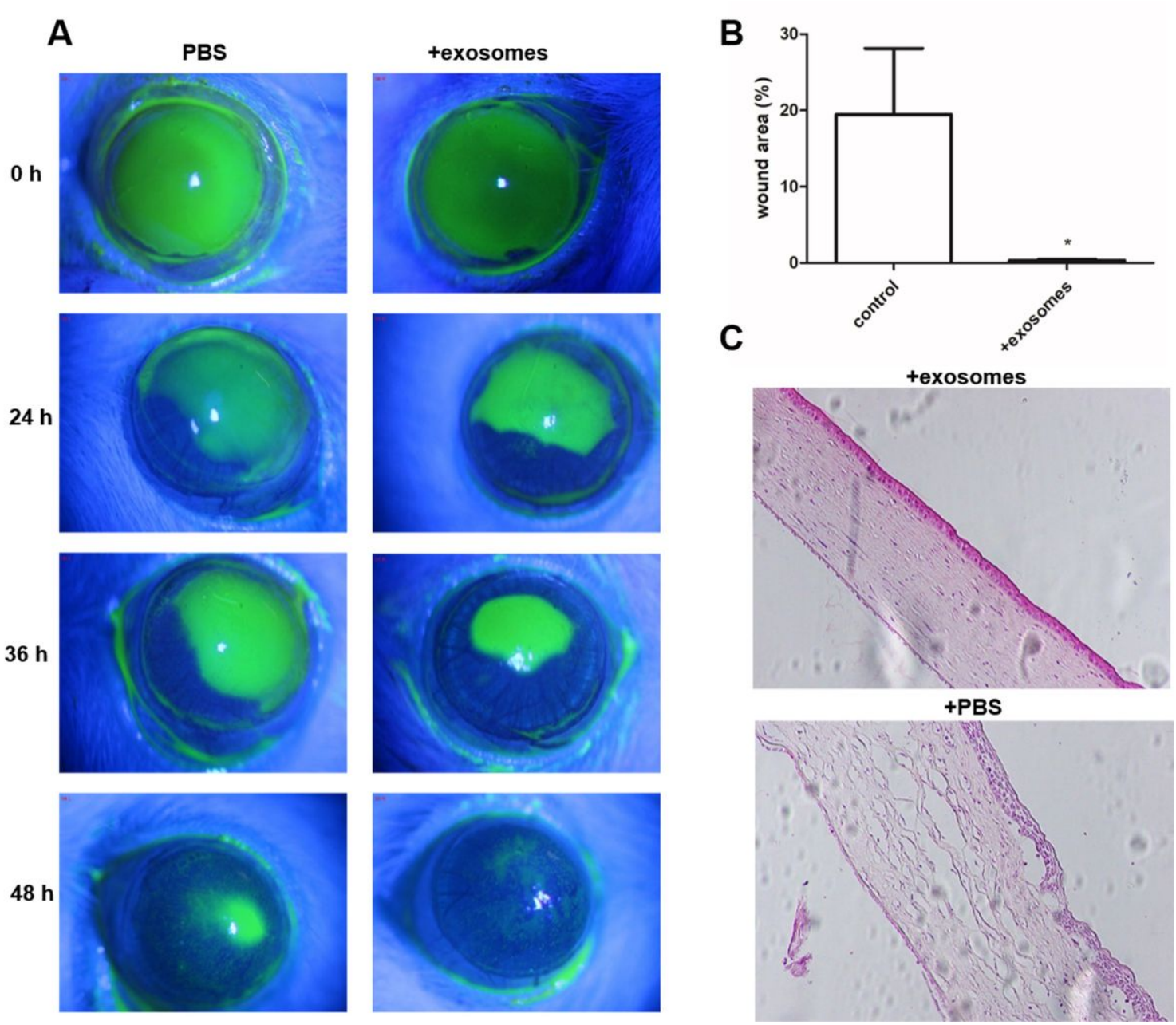

Figure 2

The effect of HUMSC-exosomes on corneal epithelial wound healing in vivo. a, b Fluorescein-stained images of defect corneas, before and after treatment with HUMSC-exosomes or PBS. Wounds treated with HUMSC-exosomes had healed significantly more than control. c H&E staining showed the histologic appearance of the cornea in exosome-group mice and PBS-group mice at 72 h. Data are expressed as the means ± SD. *P < 0.05.

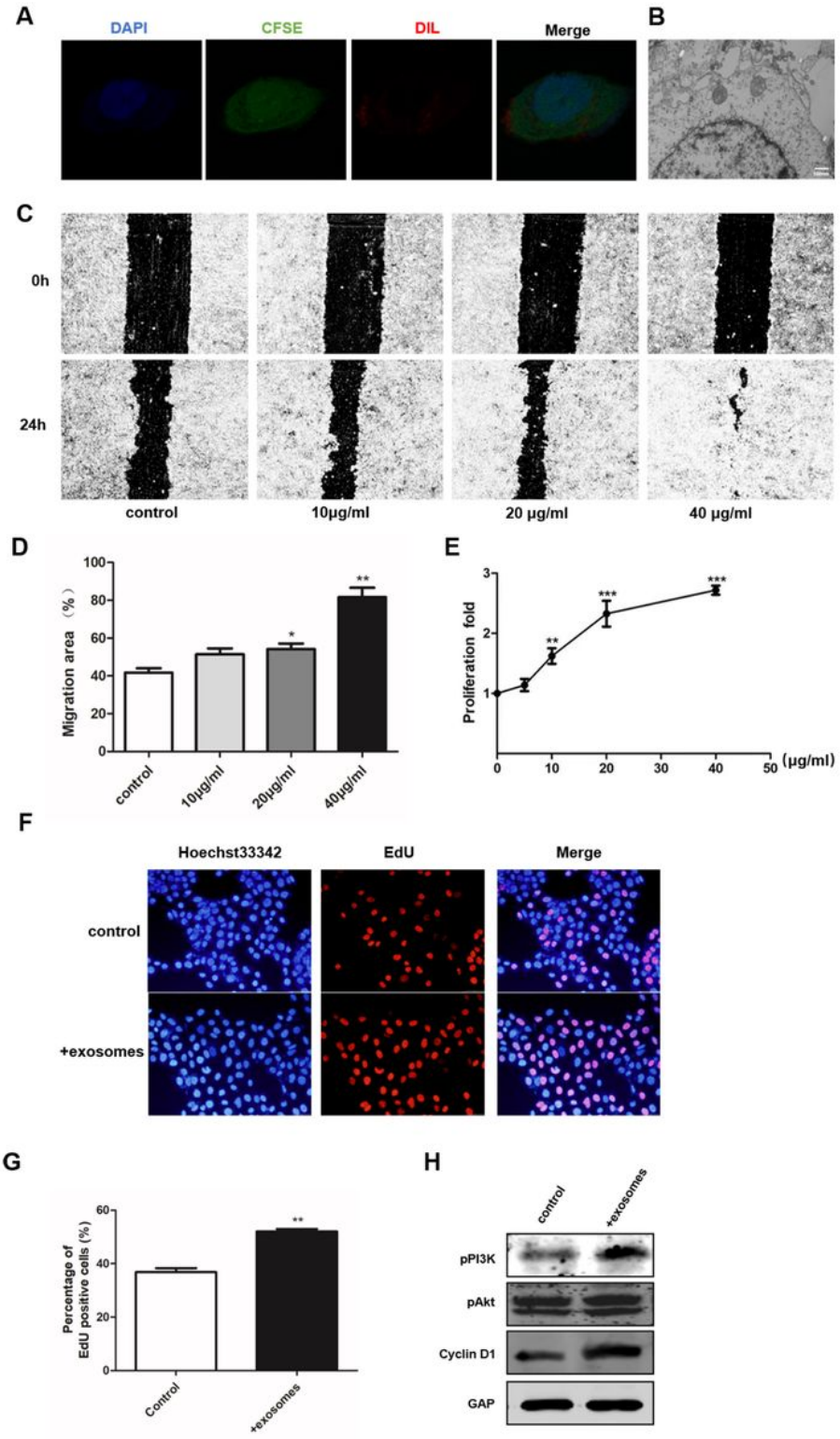

Figure 3

The effect of HUMSC-exosomes on HCECs proliferation and migration in vitro. a Fluorescence images of CFSE-labelled HCECs (green) incubated with Dil-labelled HUMSC-exosomes (red). Nuclei were stained with DAPI (blue). b TEM of HCECs incubated with HUMSC-exosomes. c, d Representative images from in vitro scratch wound healing assays demonstrating that cell migrate into the cell-free region is significantly accelerated in the presence of HUMSC-exosomes when compared to controls. e CCK-8 assay

showed increased proliferation of HCECs incubated with HUMSC-exosomes after 48 hours. f, g The proliferating HCECs was detected by EdU incorporation. The cells were treated with HUMSC-exosomes or blank control. Blue: nuclear staining (Hoechst33342); Red: EdU staining. h, i The expression of phospho-PI3K, phospho-Akt and cyclin D1 were detected through Western blot. Data are expressed as the means ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001.

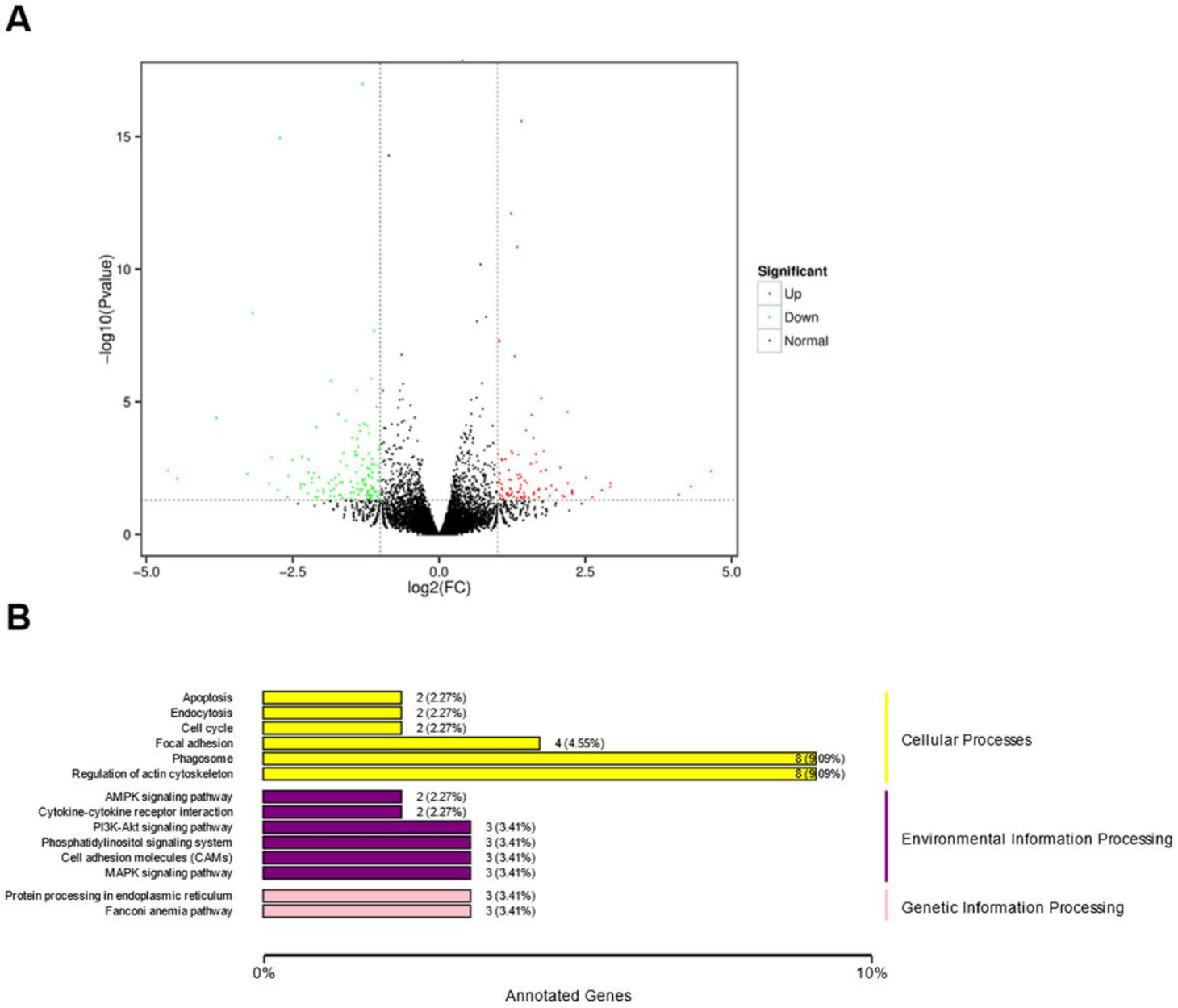

Figure 4

Transcriptome and pathway analysis of HUMSC-exosomes treatment. a Volcano Plot of DEGs between HUMSC-exosomes treated and untreated HCECs. Dots in green stands for down-regulated DEGs and red dots mean up-regulated DEGs, black dots are non-significant DEGs. b The KEGG annotation results of the DEGs were classified according to the pathway types in KEGG. DEG, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes

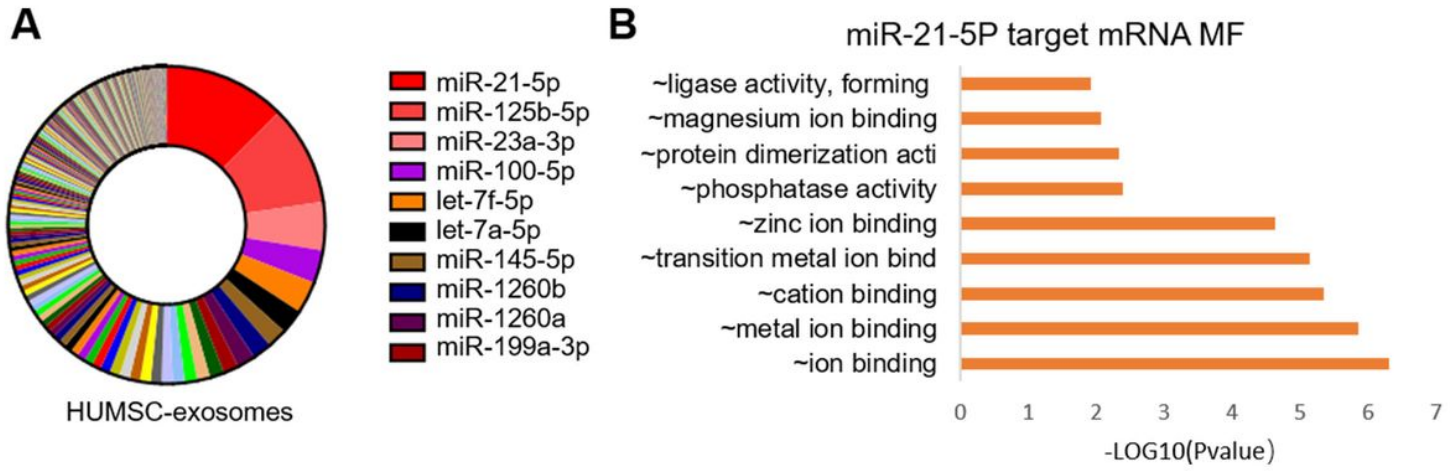

Figure 5

Identification of HUMSC-exosomal microRNAs. a miRNAs abundance analysis of HUMSC-exosomes. b mRNA targets for the microRNAs significantly enriched in HUMSC-exosomes were identified and GO analysis. MF, molecular function; GO, Gene Ontology

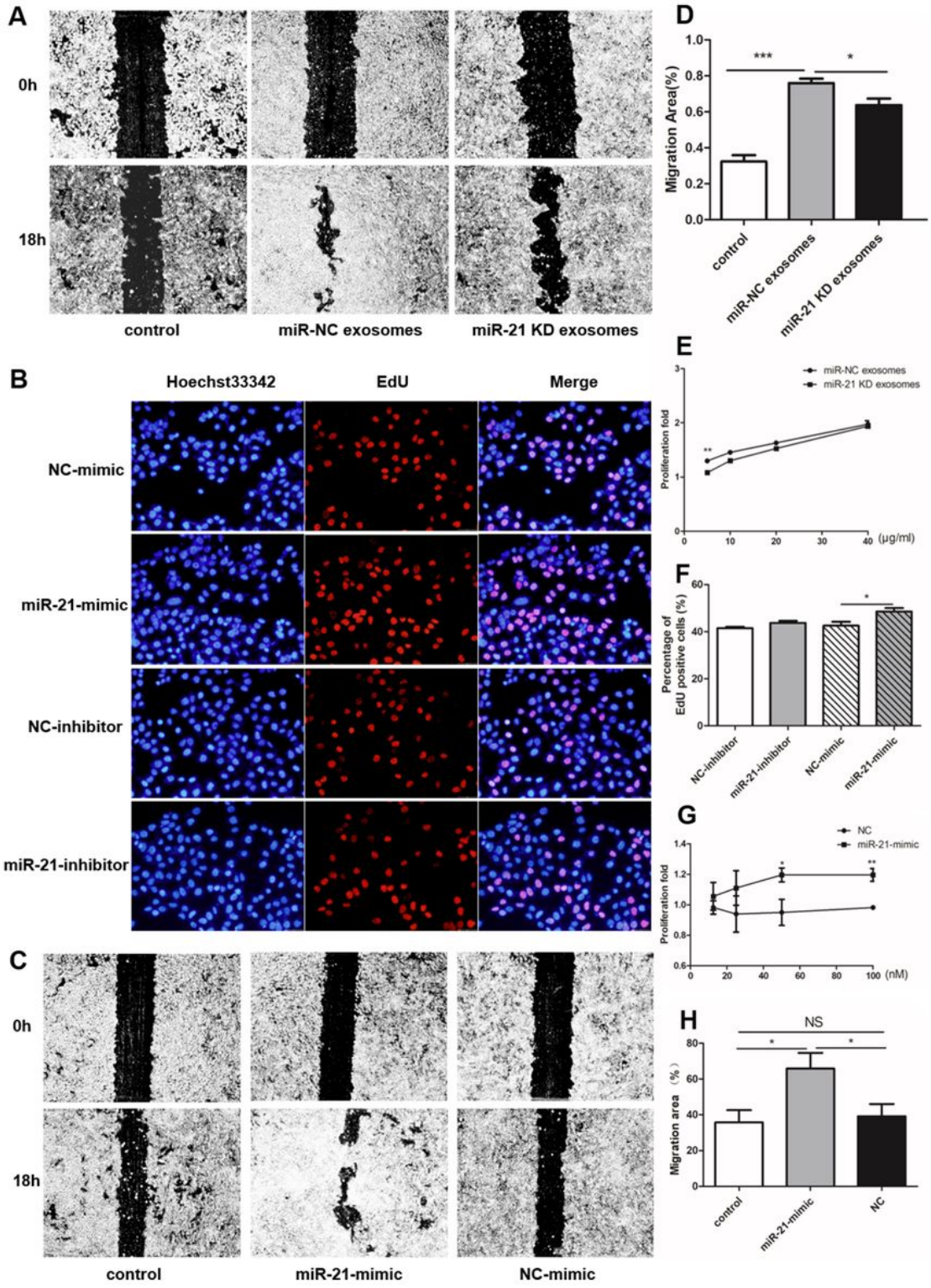

Figure 6

miR-21 involved in the process of HUMSC-exosomes promote cell proliferation and migration. a, d HCECs were treated with miR-21KD HUMSC-exosomes or miR-21 contained HUMSC-exosomes for 18h. The scratch assay showed the healing of the miR-21KD HUMSC-exosomes treated group was slower than the miR-21 contained HUMSC-exosomes treated group. b, f The proliferation of HCECs was detected by EdU incorporation after transfected with miR-21 mimics (at final concentration of 50 nM). Blue: nuclear

staining (Hoechst33342); Red: EdU staining. e The CCK-8 assay showed the proliferation of the miR-21 KD HUMSC-exosomes treated group was lower than the miR-21 contained HUMSC-exosomes treated group after 18 hours. c, h The scratch assay showed significantly faster wound closure in HCECs incubated with miR-21 mimics than NC after 18 hours. g The CCK-8 assay showed the proliferation of the miR-21 mimics group was higher than control group after 48 hours. Data are expressed as the means ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001. KD, knockdown

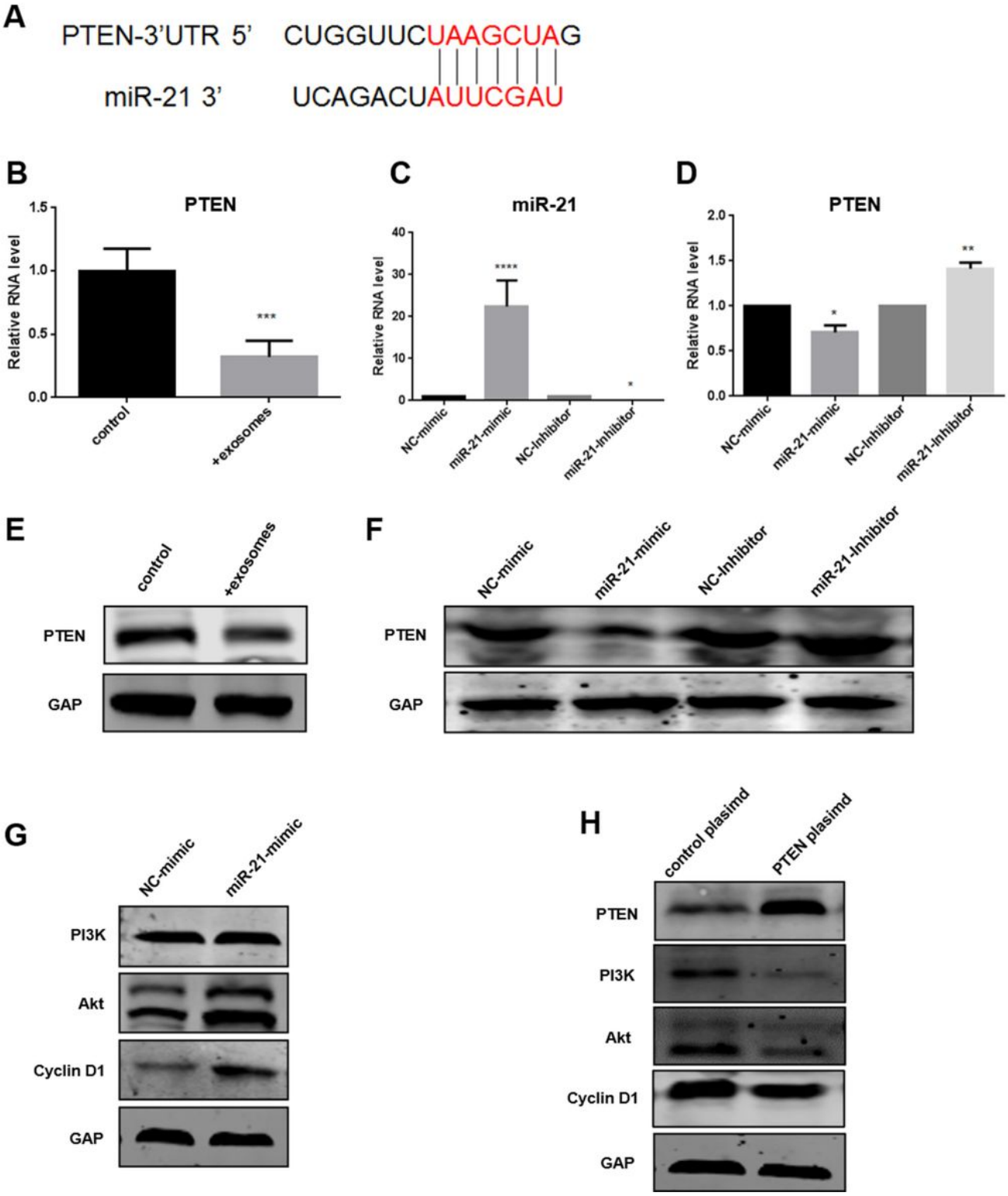

Figure 7

Exosomal miR-21 regulate HCECs proliferation and migration by activating PI3K/Akt pathway through targeting PTEN. a The binding site between miR-21 and PTEN mRNA. b, e, f HUMSC-exosomes treatment decreased the RNA and protein levels of PTEN in HCECs. c The expression level of miR-21 in HCECs. d, g, h The expression of PTEN changed with miR-21 variation. i, j The expression level of PTEN, phospho-PI3K, phospho-Akt and cyclin D1 after transfected with miR-21 mimics were detected by Western blot. k, l The expression of phospho-PI3K, phospho-Akt and cyclin D1 after overexpression of PTEN. Data are expressed as the means ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001.

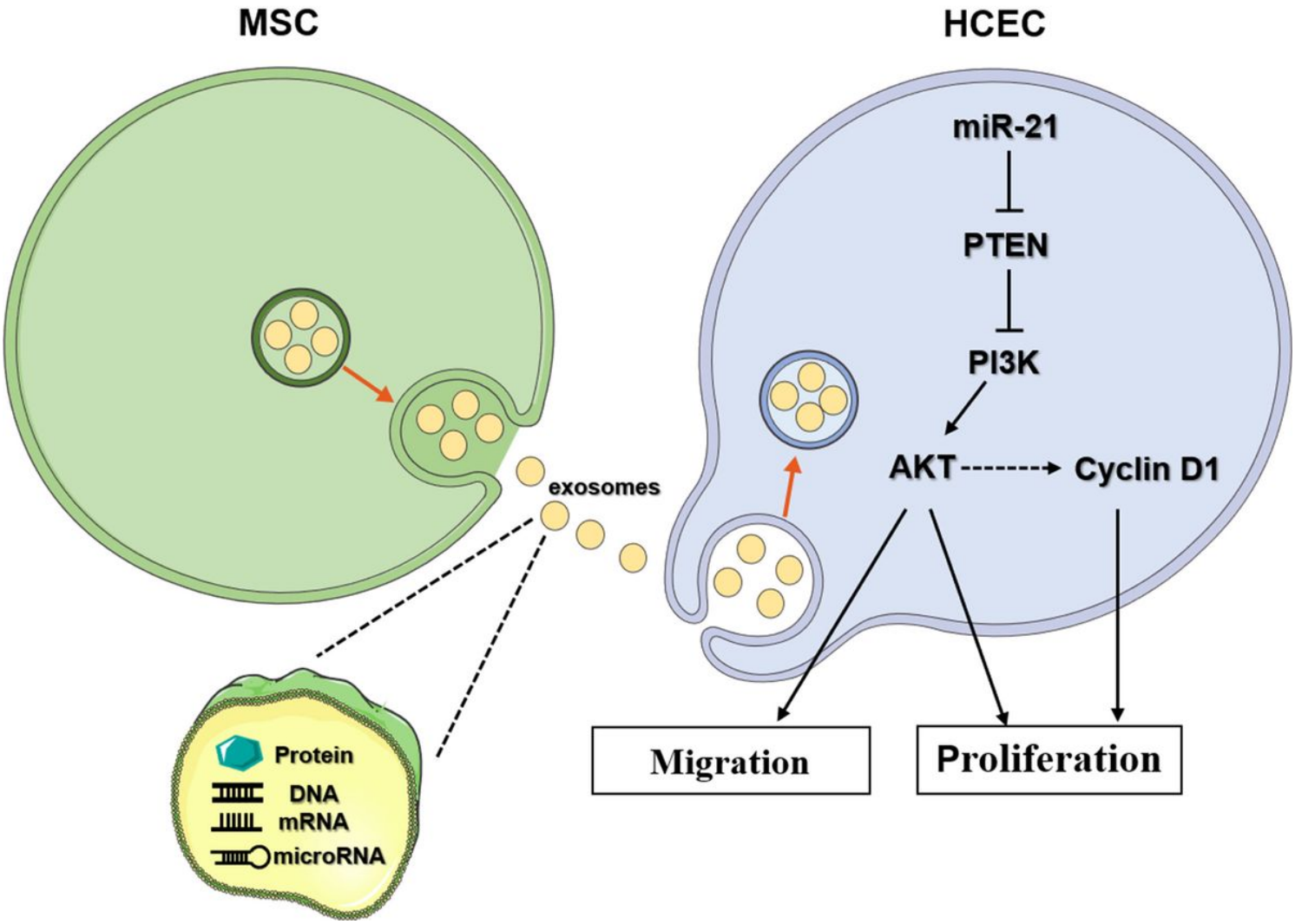

Figure 8

Schematic diagram describes the mechanism of HUMSC-exosomes in corneal epithelial defect.