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Research Article

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Umbilical cord mesenchymal stem cells-derived exosomes deliver miR-21 to promote corneal epithelial wound healing through PTEN/PI3K/Akt pathway

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Abstract

Objective: 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 mimics 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 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.

Conclusion: HUMSC-exosomes could enhance 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.

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

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

The umbilical cord was obtained from the Department of Obstetrics and Gynecology of the First Affiliated Hospital of Harbin Medical University after harvesting informed consent for research purposes, which was approved by the Ethics Committee (ethical approval number: 201859). 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). The release of EVs from HUMSCs was blocked by pre-incubating HUMSCs with 20 μM GW4869 (sigma, USA) for 24 hours. 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, pCDNA3.1-3×Flag-PTEN and empty vector plasmid as indicated.

Exosome uptake assay

For the exosome uptake analysis, 20 μg purified HUMSC-exosomes in 100 μl PBS was incubated with 1 μM Dil (Beyotime Institute of Biotechnology, China) in the dark for 30 min, washed twice with PBS, ultracentrifuged at 120,000 g for 70 min to remove nonbinding dye, and then resuspended in serum-free medium. HCECs were labeled with 5 mM CFSE in the dark for 20 min, washed twice with complete medium. 200 μl cell suspension ($5x10^4$/ml) was seeded into 35 mm glass bottom dishes (Cellvis, USA) and let the cells adhere to the glass for 12 hours. CFSE-labeled HCECs were 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 (40 μg /ml) 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 seeded 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 protease and phosphatase inhibitor (Beyotime Institute of Biotechnology, China). 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), PTEN (9188, CST), 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.

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

199 HUMSC-exosomes microRNA expression microarray GSE69909 were downloaded from GEO
200 database. Target Scan, mirBase and miRDB were used to predict the target genes of miRNAs
201 enriched in exosomes. All the predicted targets have target prediction scores ≥80 were subjected to
202 gene ontology (GO) analysis to investigate the underlying mechanism of the potential HUMSC-
203 exosomes miRNA and the target mRNAs during corneal re-epithelialization.
204
205 **Full-length transcriptome sequencing**
206 HCECs (2.5×10^6 cells) were treated with 40μg/ml HUMSC- exosomes for 48 hours, same volume
207 of PBS was added as control with three biological replicates. Total RNA was isolated
208 using the TRIzol reagent according to the manufacturer's instructions. 1μg total RNA was prepared
209 for cDNA libraries using cDNA-PCR Sequencing Kit (SQK-PCS109) protocol provided by Oxford
210 Nanopore Technologies (ONT). Briefly, the template switching activity of reverse transcriptase
211 enrich for full-length cDNAs and add defined PCR adapters directly to both ends of the first-strand
212 cDNA. And following cDNA PCR for 14 circles with LongAmp Tag (NEB). The PCR products
213 were then subjected to ONT adaptor ligation using T4 DNA ligase (NEB). Agencourt XP beads was
214 used for DNA purification according to ONT protocol. The final cDNA libraries were added to FLO-
215 MIN109 flowcells and run on PromethION platform at Biomarker Technology Company (Beijing,
216 China). KOBAS software was used to test the statistical enrichment of differential expression
217 transcripts in KEGG pathways.
218
219 **Corneal mechanical wound model and treatment**
220 The experimental protocols were approved by The Ethics Committee of First Affiliated Hospital of

Harbin Medical University (ethical approval number: 2020100). Weight from 160–180g male Sprague-Dawley rats (6-8 weeks old) were purchased from the animal experiment center of the Second Affiliated Hospital of Harbin Medical University. The rats were anesthetized with intraperitoneal injection and applied topically 0.5% proparacaine, the corneal epithelium was removed up to the corneal/limbal border with 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.

Rats were randomly divided into four groups and subconjunctival injected with 100 μl PBS containing 2×10^6 HUMSCs, equal amount of HUMSCs pretreated with GW4869, 40 μg HUMSC-exosomes or an equal volume of PBS, respectively. 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 ±

243 standard deviation (SD). Student *t*-test was used to determine statistically significant differences
244 between samples. When multiple comparison analyses were required, statistical significance was
245 evaluated by one-way ANOVA. All *P*-values <0.05 were considered statistically significant.
246

247 Results

248 1. Identification of HUMSCs and HUMSC-exosomes

249 Results of flow cytometry analysis confirmed the presence of positive expressions of typical MSC
250 makers CD105, CD90, CD73, while the surface markers of hematopoietic cells such as CD34,
251 CD11b, CD19, CD45 and HLA-DR were fairly weak to detect compared with the isotype control
252 (Fig. 1(a)). In addition, according to inverted microscopic observation, the morphology of the cells
253 was regular long spindle with directional arrangement, and presented a typical spindle shape, which
254 grew as whirlpool or cluster (Fig. 1(b)).
255

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

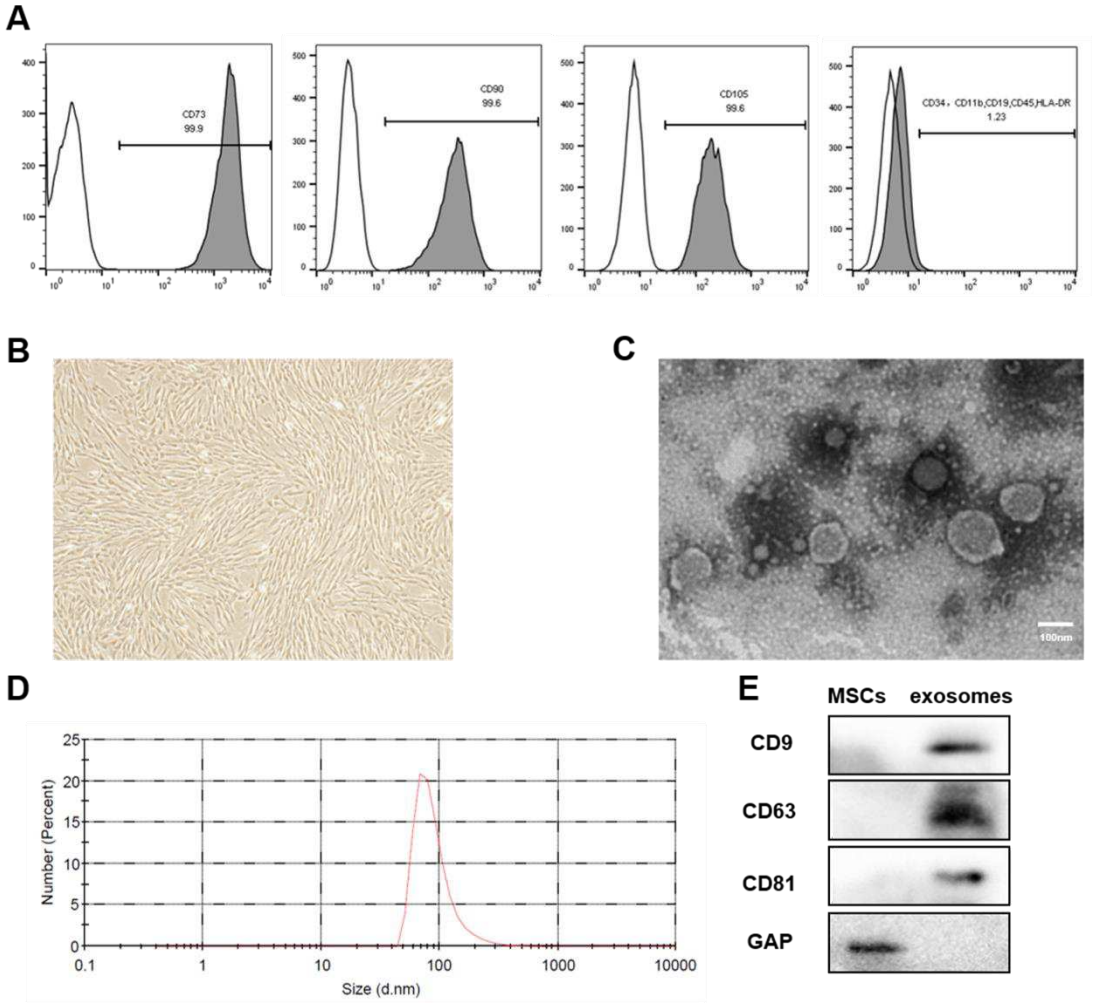


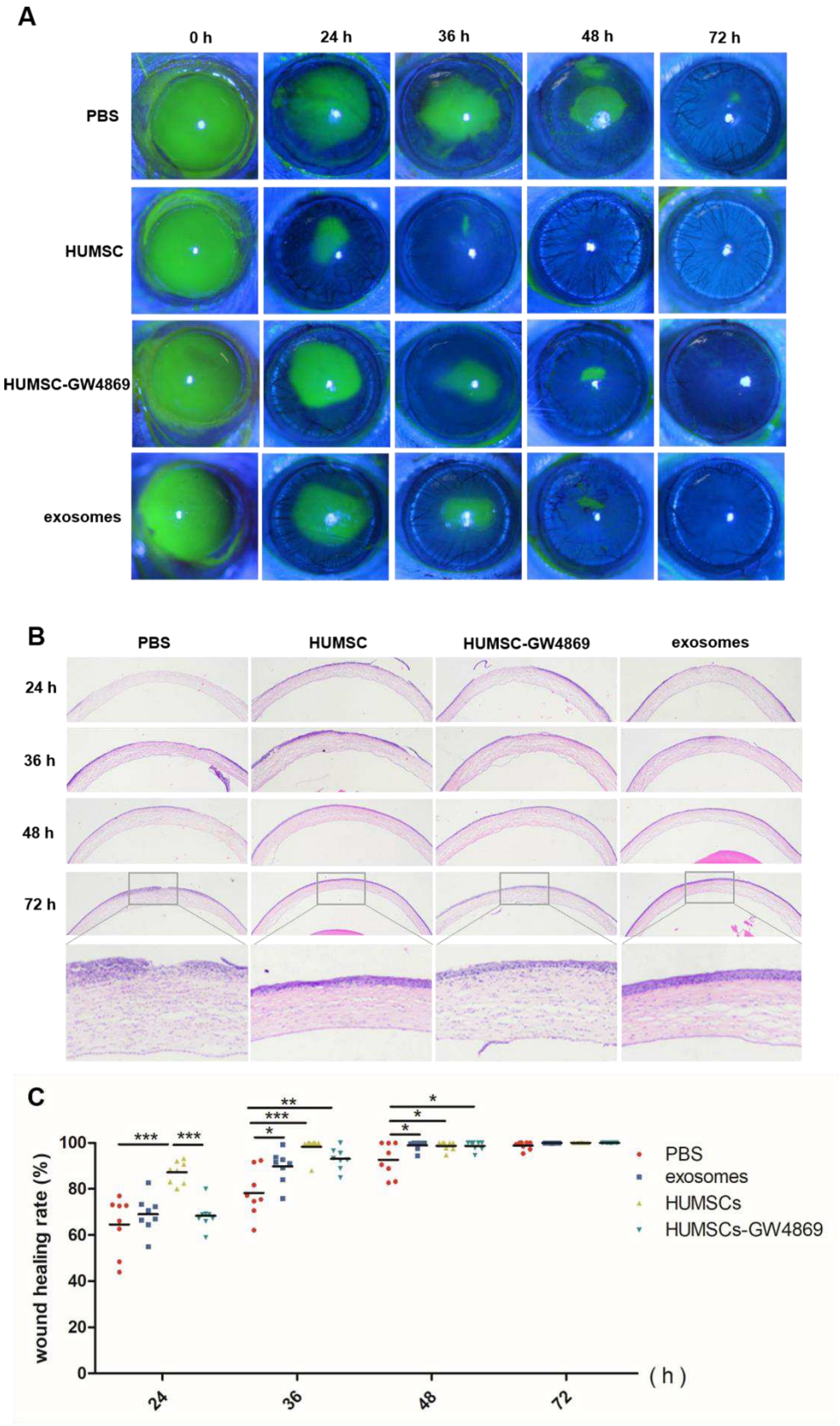
Figure. 1

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.

2. Application of HUMSCs or HUMSC-exosomes promotes corneal wound healing in a rat model

We found that subconjunctival injection of HUMSCs or HUMSC-exosomes can effectively promote the healing of corneal defects in rats, while inhibition of exosome secretion by GW4869 can

attenuated HUMSCs mediated benefits at 24 hours (Fig. 2(a) and (c)). The injured corneas treated with HUMSC and HUMSC-exosomes regained more regular arrangement and compact structure than those treated with PBS through assessing corneal tissues microstructure by H&E staining (Fig. 2(b)).



277

278 Figgure. 2

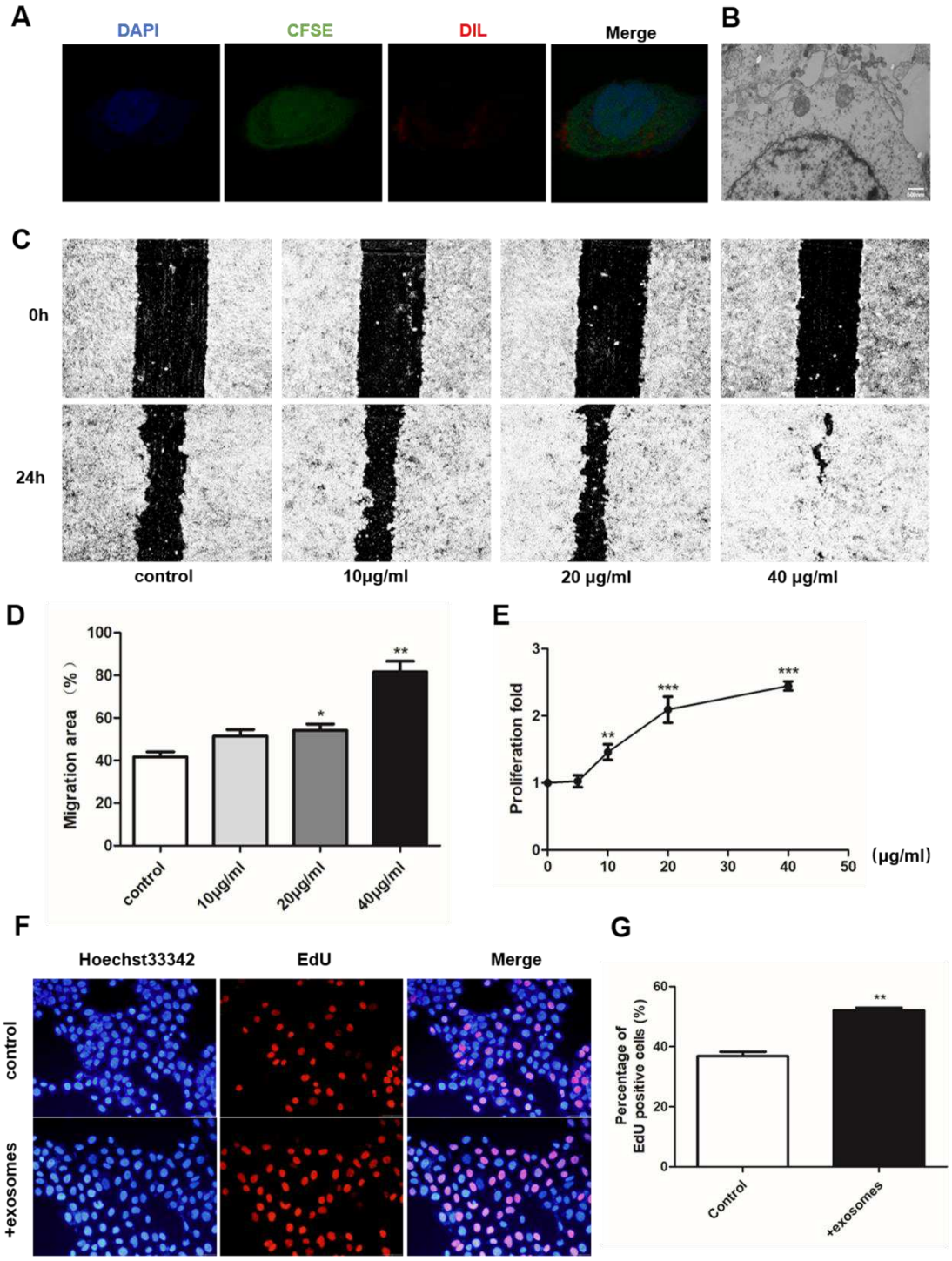
279 The effect of HUMSCs and HUMSC-exosomes on corneal epithelial wound healing in vivo. (a, c) Fluorescein-

stained images of defect corneas, before and after treatment with HUMSCs, HUMSCs-GW4869, HUMSC-exosomes or PBS. (b) H&E staining showed the histologic appearance of the cornea. Data are expressed as the means ± SD. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, $n = 8$.

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. The red nanoparticles represent the labeled exosomes that occurred in smaller clusters and were observed either surrounding the cell membrane or within the cytoplasm in HCECs. Localization results showed that exosomes derived from HUMSCs had been taken up by HCECs with the dye distributing within in the cell (Fig. 3(a)). In addition, the fusion process was also observed by TEM (Fig. 3(b)).

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. 3(c) and (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. 3(e)). 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. 3(f) and (g)).



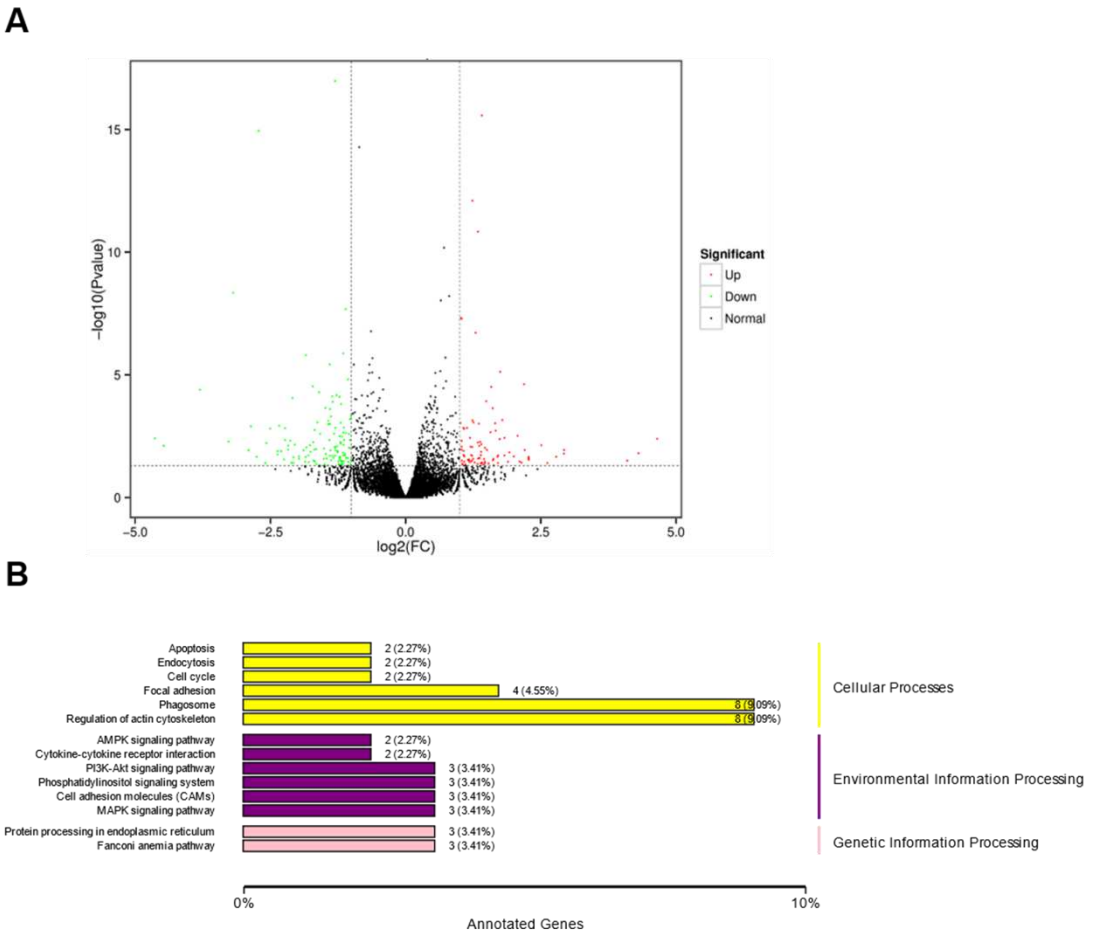
Figgure. 3

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 promoted in

the presence of HUMSC-exosomes when compared to controls, $n = 4$. (e) CCK-8 assay showed increased proliferation of HCECs incubated with HUMSC-exosomes after 48 hours, $n = 5$. (f, g) The proliferating HCECs was detected by EdU incorporation. The cells were treated with HUMSC-exosomes or blank control, $n = 3$. Blue: nuclear staining (Hoechst33342); Red: EdU staining. 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. 4(a)). 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 pathway, the phosphatidylinositol signaling system, cell adhesion molecules and MAPK signaling pathway had a significant difference between before and after HUMSC-exosome treated HCECs (Fig. 4(b)). 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.



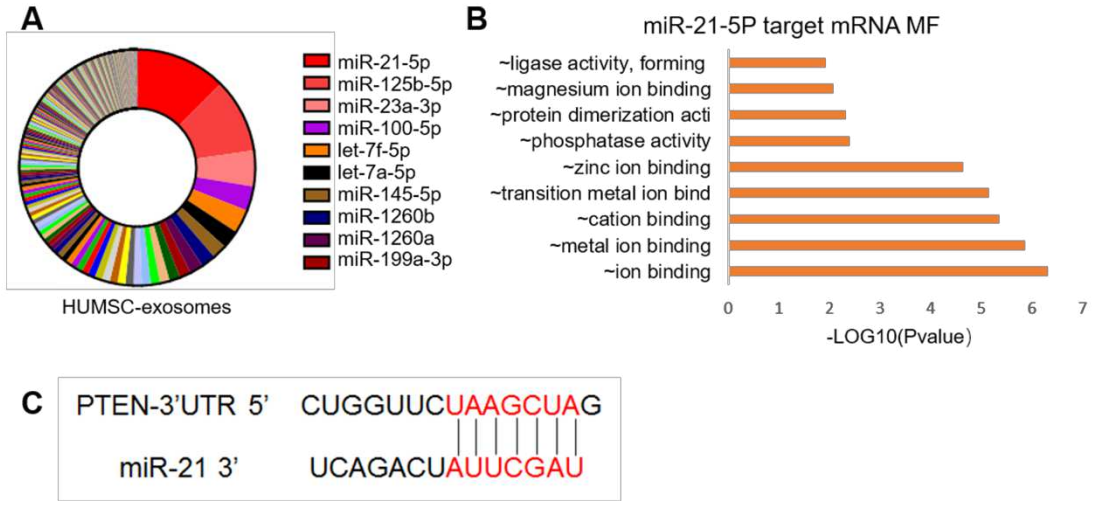
Figgure. 4

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

5. Exosomal miR-21 regulates the PTEN/ PI3K/AKT signaling pathway

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. 5(a)). 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. 5(b)). Among them, the phosphatase activity may involve in the regulation of PI3K/Akt. 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. 5(c)).

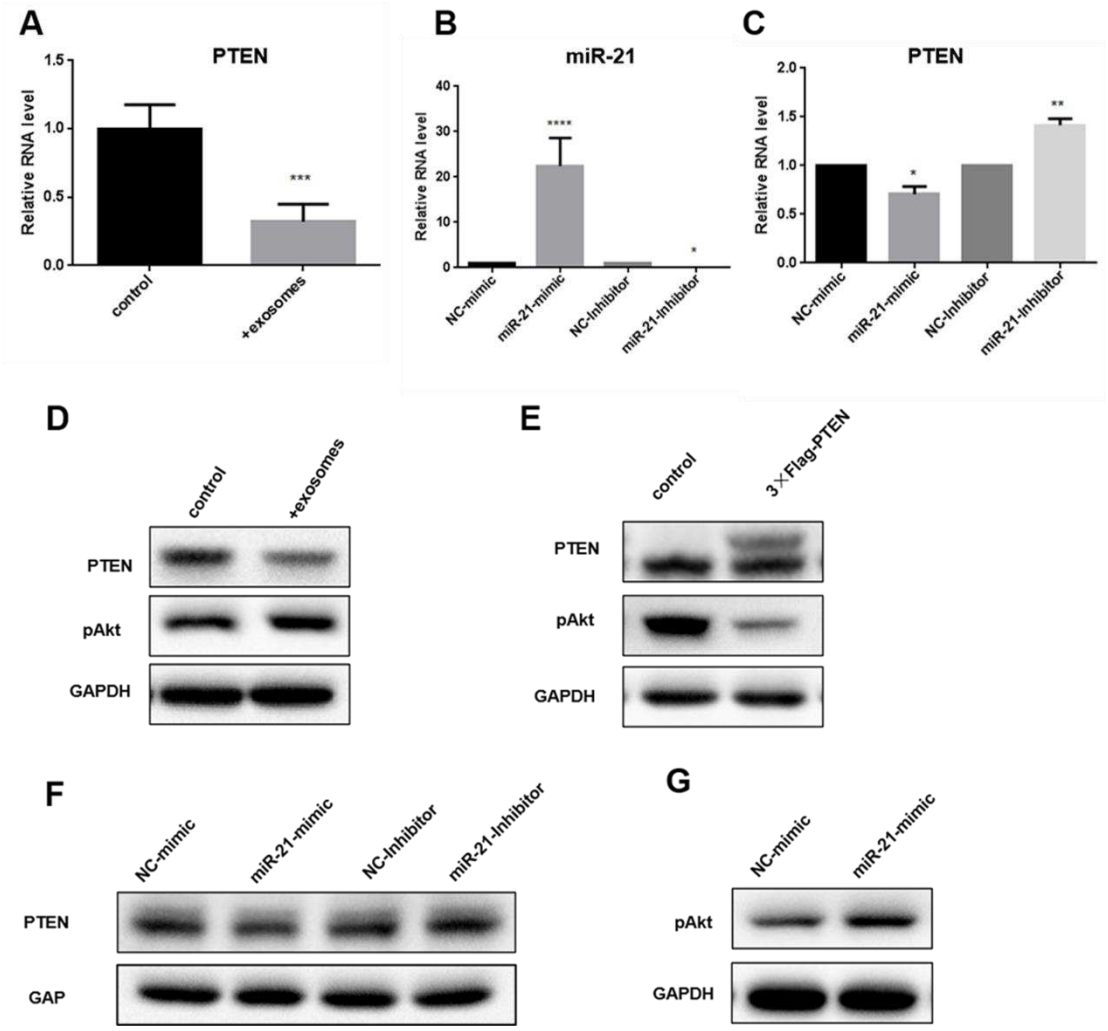


Figgure. 5

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. (c) The binding site between miR-21 and PTEN mRNA. MF, molecular function; GO, Gene Ontology

Unsurprisingly, we found that reduced mRNA and protein expression levels of PTEN were identified within HCECs after treated with HUMSC-exosomes (Fig. 6(a) and (d)). The activation of

the PI3K/Akt pathway in HCECs following HUMSC-exosome stimulation was verified by assessing the phosphorylated Akt levels (Fig. 6(d)). 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. 6(f)), and the difference was also detected in transcription level (Fig. 6(b) and (c)). We also found that the effects of miR-21 on the Akt phosphorylation were stimulative (Fig. 6(g)). Meanwhile, overexpression of PTEN down-regulated the phosphorylated Akt levels, which was important for proliferation and migration (Fig. 6(e)). These results suggested that miR-21 regulate PTEN within HCECs via post-transcriptional modify.



Fgure. 6

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

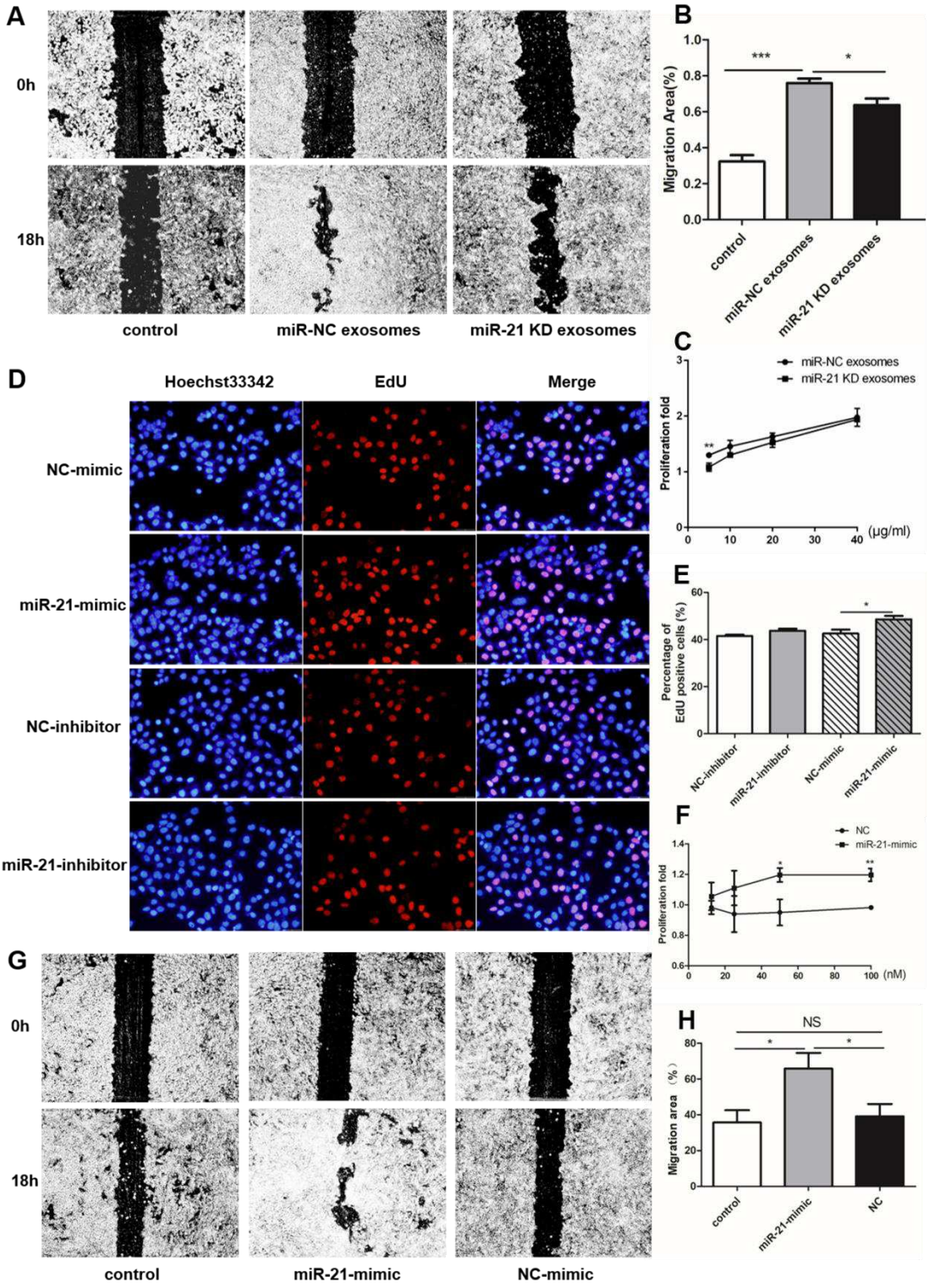
6. HUMSC-exosomes promote HCECs proliferation and migration through miR-21

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. 7(a) and (b)), as well as proliferation (Fig. 7(c)) 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. miR-21 mimics transfection significantly promoted the proliferation of HCECs compared with the NC group (Fig. 7(d)-(f)). In addition, the ability of HCECs transfected with miR-21 mimics to regain monolayer integrity was raise compared with NC-transfected cells (Fig. 7(g) and (h)).

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



Fgure. 7

miR-21 involved in the process of HUMSC-exosomes promote cell proliferation and migration. (a, b) 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, $n = 5$. (c) 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, $n = 3$. (d, e) 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, $n = 3$. (f) The CCK-8 assay showed the proliferation of the miR-21 mimics group was higher than control group after 48 hours, $n = 3$. (g, h) The scratch assay showed significantly faster wound closure in HCECs incubated with miR-21 mimics than NC after 18 hours, $n = 5$. Data are expressed as the means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. KD, knockdown

7. Schematic diagram

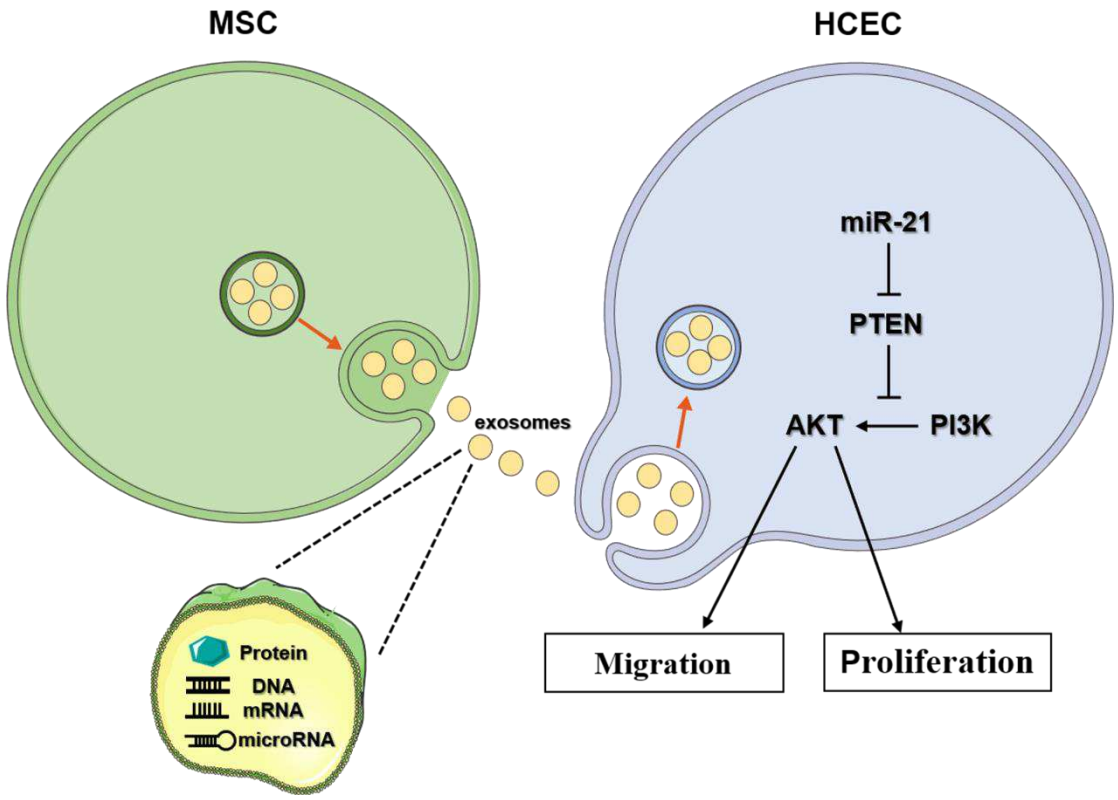


Figure. 8

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. 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.

As a cell-based therapy for treating human diseases has gained increasing interest over the last few decades，hundreds of clinics or clinical trials using human MSCs have carried out, and showed that the application of MSCs can enhance wound healing[22] and ameliorate fibrosis[23]. In this study, we have shown that subconjunctival injection of HUMSCs facilitated corneal epithelial wound healing. However, several recent studies suggest that the therapeutic effects of MSCs may

be largely mediated by paracrine effects involving proteins/peptides, hormones and vesicles packaging various molecules[24]. Among such effectors, exosomes are considered to be the key effectors to exert therapeutic function. In order to further confirm if HUMSCs paracrine effects dominate the process of cornea wound healing, HUMSCs were pretreated with GW4869, a exosomes generation blocker[25]. Expectedly, HUMSCs were less effective in enhancing wound healing when the release of exosomes was blocked. These results implicating the function of HUMSCs particularly rely on exosomes release into the microenvironment.

Researches have shown that independent application of MSC-exosomes can also play a critical role in promoting the repair of damaged tissues [26]. Moreover, MSC-exosomes have many advantages over MSCs, such as less safety concerns [27], long-term preservation and easy transportation [28], lower immunogenicity [29], capacity to cross biological barriers [30]. Previous studies have shown that exosomes from corneal MSCs can reduce scar formation and increase the transparency of corneal healing [31]. Exosomes from placental MSCs can reduce the inflammatory response during corneal alkali burn and promote the restoration of normal corneal structure [32]. Exosomes from bone marrow MSCs promoted survival of retinal ganglion cells and regeneration of their axons through miRNA-dependent mechanisms[33]. In present study, for the first time, we proved that HUMSC-exosomes could promote the repair of corneal epithelium integrity and 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 [34]. 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 [35, 36]. 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 [37]. Many studies have validated the role of miRNAs in exosomes in various types of cells [38, 39]. 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. miR-21 is a proliferation-related miRNA, and its role in wound healing was demonstrated in skin wound models [40] and cornea wound healing [41]. Despite previews results proved miR-21/SPRY2 axis participated in modulating epithelial phenotypes, promoted the migration of corneal epithelial cells and enhance the wound healing process, the mechanisms underlying miR-21 effect on corneal epithelial wound healing remain largely unknown.

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 [42].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 [43, 44]. Once the PI3K/Akt Signaling pathway was suppressed, corneal epithelial migration was delayed [45-47]. 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 [48-50]. 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 [51]. Recent studies have shown that PTEN is involved in the pathological mechanism of myocardial injury and neurocognition, also in regulating corneal epithelial defects [47, 52-54]. In addition, PTEN remains the main negative regulator of PI3K/Akt signaling through its phosphoinositide phosphatase activity [55].

To confirm the relationship among miR-21, PTEN and PI3K/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. These results demonstrated that miR-21 promoted HCECs proliferation and migration by regulating PI3K/Akt via PTEN.

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. In addition, as the situation of patient with severe corneal epithelial defect is much more complex than that in animal models, whether HUMSC-exosomes can promote the healing of severe corneal injury in clinical practice remains unknown. 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.

Conclusions

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 better corneal wound repair and regeneration. Our results offer a novel therapeutic agent for the treatment of a corneal wound as a cell-free therapy.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflict of interest regarding the publication of this paper.

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Authors' Contributions

Xiaolong Liu and Xuran Li contributed equally to this work.

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