Effect of Tear Derived Exosomes on Corneal Epithelial Cells During Diabetic Keraropathy

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Research Article

Keywords: exosomes, diabetes, cornea, microRNAs, different expression

Posted Date: March 21st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1444975/v1

Abstract

Background: As reliable intercellular transporters, exosomes for the diagnosis and function of various disease are fully explored. Also, exosomes extracted from tear film have been used as indictors for various eye disease, even systemic disease due to the easy and non-invasive collection nature with microRNAs variation. However, there still no reports focus the change and function of on exosomes and exosomal microRNAs during diabetes ocular dysfunction.

Methods: Paired diabetes and health tear film-derived exosomes were collected by test strips and identified according standard regimens. Different expression microRNAs were profiled by microarray. The function of exosomes to cornea epithelium was detected by cornea wound model in vivo and cell viability and migration assay in vitro. Bioinformatics analysis, including GO and KEGG were conducted to explore the potential biological signaling pathway.

Results: We confirmed diabetic tear film derived exosomes contribute to delay cornea wound healing and aggravate diabetic dry eye-like syndrome. Total 12 differential expression microRNAs were confirmed compared with paired controls, may participate in glycosphingolipid biosynthesis; mucin type O-Glycan biosynthesis; Ras signaling pathway; AMPK signaling pathway; bacterial invasion of epithelial cells; ErbB signaling pathway; cGMP-PKG signaling pathway

Conclusion: This study provides evidence that diabetic tear film derived exosomes can affect the ocular surface health during diabetes, while healthy exosomes have no similar effects. Thus, the different expression of microRNAs existed in the exosomes from diabetes and healthy volunteer may be the reason of different function.

Introduction

Tear fluids (TF) are rich in protein, lipids, lysozyme, lipocalin and other metabolites and play a significant role in corneal health and immune protection when applied evenly on the surface of the eye.

In human and animal diabetes, the release of inflammatory factors and accumulation of AGEs in tear were higher than the normal due to the activation of inflammation, hyperglycemia-related oxidative stress, and hyperglycemia-related osmotic pressure. Also, the exposure of the corneal epithelium to TF has been found increase certain inflammatory/immune cytokines, abnormal differentiation, accelerated detachment and apoptosis [1].

Exosomes are nanoparticles range from 30 to 150nm which can be found contained in various body fluid, such as plasma [2], tears[3] and aqueous humor [4], numerous researches have reported that exosomes can mediate signal transduction, modulate immune reactions, and participates in intercellular communication by transferring proteins, lipids, miRNAs and mRNAs included.

As the most abundant composition included in exosomes, there are significant different expression profiles of exosomal microRNAs in breast cancer[5], glioma[6]. Beyond being a biomarker, exosomal microRNAs have been reported to contribute to cardiac dysregulation in chronic heart failure [7], protect against acute lung injury [8].

Several studies have showed that tear film -derived exosomes can be considered as a potential, diagnostic, specific biomarkers for the diagnosis of primary Sjogren syndrome [9, 10], primary open-angle glaucoma [11, 12], and thyroid eye disease [13], even prostate and breast cancer [14, 15].

However, there still no studies focus on investigating or characterizing diabetic tear film (DTF) derived exosomal RNAs, also the role involved in ocular surface disease.

Herein, we isolated exosomes from patients' tear films with diabetes mellitus (Dia-EVs) or healthy people to investigate different expression change of miRNAs contained in exosomes that may be relative to ocular surface complication in this study,

Methods

Participant Recruitment and Collection of Tear Fluid

The whole research procedure was performed following the Declaration of Helsinki and gain approval from the ethical committee of Harbin Medical University and sign consent by the subjects after explanation of the nature and possible consequences of the study

DM patients were recruited from the first hospital of Harbin Medical University.

Tear fluid was collected with using test strips (5*50mm) without topical anesthesia. Examiners placed the test strip at the lateral eyelid margin after wearing gloves, avoiding contact with the eyelid or bulbar conjunctiva.

The participants keep their both eyes close for 5 minutes. Once remove the strip, and the wetted part was then stored in a cuvette containing 200 mL phosphate buffered saline (PBS), subsequently placed in a deep freezer at − 80°C.

Exosome purification and identification

Briefly, tear sample were placed on the ice and diluted with precool PBS. To remove debris and the impurities from samples, then all samples from diabetes or healthy people were centrifuged at 2,000g at 4˚C for 15 minutes. Subsequently, the exosomes were precipitated following manufacturer's recommendations (4484453, Invitrogen™, Carlsbad, CA). Finally, the collected exosomes were resuspended with PBS for further use.

BCA protein assay kit was used to determine the concentration of exosomes (Beyotime, China) as suggested by the manufacturer. Transmission electron microscopy (TEM) was used to observe the morphology (Tecnai G2 Spirit BioTwin, USA).

Dynamic Light Scattering (DLS) was used to measure the size distribution. Finally, the protein marker within exosomes membrane were detected by Western Blot as the following description.

Western Blotting

Total protein extracted from exosomes was lysed with RIPA contained protease inhibitor, phosphatase inhibitor after washed with cold PBS twice, supernatants were collected by centrifuging at 10,000g for 20 minutes, and the concentration was quantified by BCA method. Interest proteins was separated by electrophoresis at 80v for SDS-PAGE and then transferred to PVDF membranes, 5% non-fat milk dissolved in Tris-buffered saline with Tween-20 was used to block for 1h at room temperature, then the membrane was incubated with primary antibodies against CD63 (ab59479, Abcam), CD81 (00679767, Invitrogen) overnight at 4°C. after membrane was washed and incubated with secondary antibody that horseradish peroxidase (HRP)-labeled (Abcam, UK). Proteins were visualized by the enhanced chemiluminescence system (Alpha Innotech, USA).

miRNA Microarrays

Exosomes were extracted from DM patients and health, and exosomal total RNAs were stored at cryogenic refrigerator for further microarray analysis.

According to the manufacturer's recommendations, labeled miRNA was hybridized and scanned by with miRNA Microarray System. Next, Raw data were normalized and analyzed with R to determine the different miRNAs expression.

Bioinformatics analysis

Online tool was utilized explore different miRNA functions, analysis the potential targets of interest miRNA and then identify probable pathway for further analysis (http://www.microrna.gr/miRPathv3, [16])

Cell culture and treatment

The human corneal epithelial cells line was obtained from commercial corporation (HCECs, BNCC, BNCC337876, China), HCECs were cultured in DMEM/F12 medium contained 10% fetal bovine serum, 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). Cells were refreshed total medium every 2–3 days

In Vitro Scratch Wound Assay

Scratch wound healing assay Scratch wounding of cultured confluent corneal epithelial cells was performed as previously described. Briefly, HCECs were grown to confluence on 12-well plates. The monolayer was wounded using the tip of a sterile 200-μl pipette. The cells were washed twice with PBS to remove the floating cells, and then cultured in serum-free culture medium containing exosomes at the indicated concentration. The wound area was recorded photographically at different timepoints using phase-contrast microscopy and was measured using ImageJ software.

In Vitro Cell Proliferation Assay

The cell viability of HCECs was detected by Cell Counting Kit-8 (CCK-8) assay following manufacturer guidance. HCECs were seed in a 96-well plate and treated with 20μg/ μL exosomes or vehicle control for 24 hours after cell adhesion. Before detecting the absorbance, the CCK-8 solution (1:10) was added at appropriate check point and cultured with cells at atmosphere at 37 ◦C for additional 2 h. The absorbance was determined by a microplate reader, the wavelength was set at 450 nm (680; Bio-Rad, Hercules, CA). The cell activity is calculated as percentage of control cells (100%) with averages from six replicates.

Corneal abrasion and exosomes Treatment

The experiment protocols were approved by the animal ethical committee of Harbin Medical University. Whole corneal abrasion was performed as previously described[17], age from six to 6weeks C57BL/6J mice were anesthetized. Corneal epithelium was removed by scraper under a dissecting microscope after topical anaesthetic with 0.5% proparacaine.

exosome suspension (total 20ug) or the PBS, served as a control were topical application by subconjunctival injection. The comparison between exosomes and vehicle was monitored by the percentage of remaining defect and original wound area with fluorescein staining and captured with a digital camera.

the image processing software was ImageJ software.

Desiccating Stress

Age from six to eight weeks SD rats was chose. DS was conducted by keeping room humidity below 20–30% and making an airflow in front of cages for consecutive 16 days, while a relative humidity above 50–75% was considered as the control, all rats were maintained with food and water freely and raised for adaption a week [18].

Exosomes suspension extracted from diabetes or healthy was topically supplied on the right eye for four times one day that last for three days,

Corneal staining score

Corneal staining score was performed as previously described by the Cornea and Contact Lens Research Unit (CCLRU) [19].

The corneas were divided into five quadrants and scored separately, and all the scores were added up to obtain the final score. Rating scale 0: no staining; 1 point: 30 dot count; 2 points: dot count > 30 but not diffuse; 3 points: severe diffuse staining but not patchy; 4 points: patchy pigmentation.

Statistical analysis

All data are represented as SD and considered as significant statistically when p-value < 0.05.

student's t-test was utilized to determine the comparison between two groups with GraphPad Prism 6 (GraphPad ,CA)

Results

1. Characteristics of Exosomes Obtained from tear film

All participants had been examined to excluded other diseases and DM had been confirmed by HbAlc (Table1).

Table 1
the basic information of patients

Age	Gender (F/M)	HbAlc (%)
53.5 ± 10.5	13/12	9.05 ± 2.1

Transmission electron microscopy (TEM) detection showed the ultrastructurally morphologies of exosomes were cup-shaped (Fig. 1a); DLS shows the mean diameter was 120.75 ± 6.0 nm (Fig. 1b); Western blot detected the presence of known conventional exosomal markers CD81, CD63 (Fig. 1c).

Table 1The signature of diabetes patients

Figure 1 The identification of tear derived exosomes

a. The morphology of vesicles extracted from tears was determined by TEM (scale bar = 200 μm)

b. The diameter size of vesicles extracted from tears is consistent with the known size of exosomes.

c. The exosomal markers, CD6 and CD81 were detected by Western blot assay.

2. Dia-EVs deteriorate environment control induced dry eye-like ocular surface damages

Dry eye syndrome (DES) has been reported influence almost 15–33% DM patients over 65 years old cross the whole world. [20], the incidence and severity have positive related with the course of diabetes disease [21]. Tear film comprise molecules, lysozyme; metabolites play an important role in maintaining the stability of ocular surface, while tear film dysfunction, including hyperosmolarity, instability or compositions change will aggravate the feedback of corneal epithelium and surrounding tissues deterioration[22]. We asked whether Dia-EVs could be closely associated with desiccation stress-induced corneal epithelium damage, after environment humidity was controlled below 20% prior to subconjunctival injection for consecutive seven days, exosomes administration contributed to more puncate defect compared with control exosomes (Fig. 2a, c).

HE staining (Fig. 2b) showed the corneal epithelial cells were indistinctly stratified, with large cell volume, disordered arrangement and surface unsmooth. These observations make us conclude the function of our experimental exosome may be not enough for inducing diabetic dry eye, but still toxic for corneal epithelium already under stresses.

Figure 2 The different effect of tear derived exosomes on ocular surface

a. The fluorescein stain of tear derived exosomes induced corneal epithelium damage under desiccation stress.

b. HE staining of damaged corneal epithelium.

c. The statistical analysis of corneal staining (n = 8).

3. Dia-EVs delay corneal epithelial wound healing

A number of published studies have indicated the diabetic cornea exhibits a delayed wound-healing and the persistent impaired cornea epithelium defect may lead to sight-threatening complications, including ocular surface dysfunction, microbial keratitis and scarring. Various molecular, including insulin-like growth factor-binding protein 1, advanced glycation end products have been indicated to accumulation within tear [23]. Although previous studies characterize the altered corneal protein concentrations, nerve signaling have regulatory effects on wound healing process, there still no reports focus on Dia-EVs.

So we evaluate the effect of Dia-EVs on cornea wound healing in vivo by an corneal wound healing model with the method described previously. After abrasion of corneal epithelium, the cornea treated with Dia-EVs or Con-EVs, the epithelial wound was still defective at 24 hours, while completed healed in the mice after treating with Con-EVs (Fig. 3a, b), even the edema faded away (Fig. 3a, c).

Figure 3 The different recovery rate of wounded corneal epithelium under tear derived exosomes

a. The fluorescein stain in the wounded cornea.

b. The statistical analysis of wound healing rate (n = 6).

c. The statistical analysis of edema area (n = 6).

4. Dia-EVs contribute to deteriorate cornea epithelial cells proliferation and migration

Cornea epithelium defect is the most frequently exposed various damage, the rapid cornea healing not for maintain physical structure, but present as a barrier for further secondary injury, the normal process corneal healing comprises cell proliferation; migration and programmed death. While when people have already suffered diabetes, various growth factors or signal transduction have made a large alteration in tears thus delay whole cornea recovery[24].

To further clarify whether Dia-EVs contained in tears regulate corneal epithelial wound healing, HCECs were applicated separately with Dia-EVs or Con-EVs. After 24 h treatment, Dia-EVs administration resulted in a cytotoxic effect on HCEC viability, and Con-EVs has no obvious influence (Fig. 4b).

Similarly, consistent with this observation, scratch wound assay results showed exposure to Dia-EVs suppressed the migration of HCECs, the re-epithelialization of monolayers showed a time dependence (Fig. 4a, c).

Figure 4 Dia-EVs deteriorated HCEC cell proliferation and migration in vitro

a. The healing area of HCECs was quantitated by wound healing assay

b. CCK-8 assay was used to measure the proliferation of HCECs after treated with Dia-EVs or Con-EVs for 24 hours.

c. The statistical analysis of healing rate (n = 3)

5. Differential expression of miRNAs in Dia-EVs

Previously, numerous inflammatory factors, such as TNF-α, IL-4 and IL-8 were detected at higher levels in the tears of type 2 diabetic patients [25, 26], even microbiota in ocular surface get more complex in abundance and diversity distribution [13] make the different function of tear films.

To determine underlying mechanism of the different function of exosomes between health and diabetes, herein, microarray was used to determine the levels of Dia-EVs microRNAs.

Based on fold change greater than 1, a set of 12 small RNAs from Dia-EVs and Con-EVs was assessed and identified as differentially expressed. Among them, the concentrations of miR-4443 and significantly increased compared with control exosomes, whereas three miRNAs were suppressed (Table2). The heat map generated according 12 differentially expressed miRNAs illustrated the distinguishable miRNAs expression profile of the samples (Fig. 5a). The volcano plot of miRNAs those expression fold change >

2.0 was created to show the quick visual identification of miRNAs (Fig. 5b). The hierarchical cluster is constructed based on the relative abundance of miRNAs demonstrated that the DM and control groups were different (Fig. 5c).

Table 2
Total significantly differentially expressed miRNAs between Dia-EVs VS Con-EVs

Systematic Name	P values	foldchange	Regulation
hsa-miR-1254	0.011064416	0.319579453	up
hsa-miR-208a-5p	0.006692563	0.445251528	up
hsa-miR-3156-5p	0.012227109	2.040317703	down
hsa-miR-423-5p	0.037746593	0.41132038	up
hsa-miR-4443	0.001135519	0.335312367	up
hsa-miR-4687-3p	0.045887284	0.486188256	up
hsa-miR-4707-3p	0.035154498	0.489150825	up
hsa-miR-5585-3p	0.036176931	0.270891312	up
hsa-miR-6073	0.029700396	0.432553573	up
hsa-miR-6087	0.020402541	0.489614159	up
hsa-miR-671-5p	0.040076376	9.933437101	up
hsa-miR-6847-5p	0.011340659	0.379851043	up

Table 2 the different expressed genes compared between Dia-EVs and Con-EVs treatments

Figure 5 the signature of different expressed miRNAs

a. The heat map of miRNAs showed the different expression within different samples.

b. the volcano plot showed the different miRNAs change according the fold change.

c. the hierarchical cluster showed the distance contained within different expressed miRNAs.

6.The KEGG and GO analysis of different express miRNA

Numerous studies showed that miRNAs interact with the 3’ UTR of target mRNAs and induce their degradation[27], miRNAs are critical for physiological development and participate in a variety of biological processes and expression alteration are involved in many human diseases[28]

We performed bioinformatics analysis, including Gene ontology enrichment and pathway analyses with their identified target genes to explore potential function associated with differentially expressed miRNAs.

After mapping the differentially expressed miRNAs to their target genes, the top 20 most significant KEGG terms were found (Fig. 6a). glycosphingolipid biosynthesis is the highest enrichment one among all of these pathways. Functional annotation of these miRNAs was speculated by GO function analysis (Fig. 6b).

Figure 6 the bioinformatics analysis of target genes to the identified miRNAs.

a. The KEGG analysis showed the target gened participate in AMPK, Ras which related to apoptosis pathway.

b. All different miRNAs were annotated by GO analysis.

Discussion

In this study, we found and confirm there has expression change of microRNAs contained in tear film in DM patients compared with healthy controls for the first time.

We further explored Dia-EVs deteriorated corneal epithelium integrity and decrease corneal epithelium recovery though suppress HCECs proliferation and migration which according with cornea wound of DM patients has a delayed recovery[29].

Exosomes, belong to extracellular vesicles could be formed and secreted into circulation by almost all cell types in physiology and pathology situation, whereupon internalized by target cells.

Diabetic circulating exosomes has been reported affect numerous tissues, including liver, skeletal muscle, adipose tissue, and brain though blood, urine, breast milk, and cerebrospinal fluids circulation[30–33].

Approximately 70% of patients with diabetes suffer from ocular surface disorder including corneal epithelial fragility, dry eye, delay and incomplete wound repair[29] cause by the changes of tear compositions, reduced tear secretion, and oxidative stress in the hyperglycemic conditions[29, 34, 35]. The change of tear concentration can influence corneal nerve morphology [36], modulate corneal sensitivity and ocular surface integrity in diabetic patients [37]. However, the EVs contained within diabetic tears still hasn’t been reported.

In this study, Dia-EVs can cause alterations in the corneal epithelial basal cells and basement membrane, leading to superficial punctate defects and erosions, we also applied Con-EVs and Dia-EVs to the wound cornea by subconjunctival injection and monitored the impact of such treatment on overall wound healing, the observation confirm Dia-EVs significantly compromise the layers of newly generated epithelial cells and delay recovery time during the wound healing progression. The experiment in vitro investigated the influence of Dia- EVs on HCECs and found Dia-EVs disrupted corneal epithelium recovery

by impairing HCECs proliferation, migration. Our results demonstrated that Dia-EVs impaired HCECs functionality, which may explain why diabetic epithelium tends to persist once it has developed after various damage.

Diabetic circulating exosomes have been shown to be an ideal kind of biomarker used for prognosis of diseases or detection of preclinical lesion due to the liquid membrane could seclude external substance, then protected miRNAs, lncRNAs or protein from degradation. Indeed, the concentration or distribution change of miRNAs have been proved participate in initiating or reversing diabetic complication and be strong associated with the diversity and severity. For example, exosomal let-7c-5p has been confirmed related to albuminuria in patients with diabetic nephropathy [31], exosomal miR-20b-5p could rectify the impairment of insulin function [38].

In our present study, we focused on the different expression miRNAs contained the tear film derived exosomes from DM patients and healthy controls, the analysis of miRNAs profile shows a total of 20 miRNAs were confirmed, including 11 up-regulated and 1 down-regulated miRNAs. The performance of KEGG and GO help gain insight of potential function enriched in, of these pathways we revealed that the most important pathway was glycosphingolipid biosynthesis, mucin type O-Glycan biosynthesis and bacterial invasion of epithelial cells. Many studies also have indicated the role of activation of RAS[39, 40] or AMPK[41] in diabetic keratopathy. There were also several studies about the cGMP-PKG signaling pathway[42] and Insulin signaling pathway[29]

Moreover, previous studies have revealed exosomes can transfer miRNAs to proximal or distal cells [43, 44]. We found some miRNAs have been reported in previous studies, for example, the higher expression of exosomal miR-4443 from breast cancer promotes metastasis and has correlation with highly invasion [45]. The up-regulation of miR-4443 induced monocyte dysfunction by activating anti-inflammatory cytokines release though NF-κB signaling pathway [46]. Serum exosomal miR-208a is significantly up-regulated and related with clinical characteristics of acute coronary syndrome[47]. Plasma exosomal miR-423-3p can be a potential predictive biomarker for resistance prostate cancer development[48]. However, the role of Dia-exosomal miRNAs in HCECs is unknown, although this study revealed the potential function of different expression exosomal miRNAs, research is still needed to determine the definite function of circulating exosomal miRNAs.

Conclusion

In conclusion, we found that Dia-EVs suppress cornea wound repair by suppressing HCECs functionality in vivo and in vitro and miRNAs expression profiles of tear exosomes was alter between diabetic patients and healthy controls, function analysis indicated different expression miRNAs may participate in diabetic keratopathy though glycosphingolipid biosynthesis, mucin type O-Glycan biosynthesis and bacterial invasion of epithelial cells, RAS, AMPK, cGMP-PKG signaling pathway and Insulin signaling pathway

List Of Abbreviations

TF Tear fluids

DLS Dynamic Light Scattering

Dia-EVs Exosomes from patients' tear films with diabetes mellitus

TEM Transmission electron microscopy

HCECs Human corneal epithelial cells

CCK-8 Cell Counting Kit-8

DS Desiccating Stress

CCLRU The Cornea and Contact Lens Research Unit

Declarations

Ethics approval and consent to participate

The whole research procedure was performed following the Declaration of Helsinki and gain approval from the ethical committee of Harbin Medical University and sign consent by the subjects after explanation of the nature and possible consequences of the study.

For animals, the experiment protocols were approved by the animal ethical committee of Harbin Medical University and carried out in compliance with the ARRIVE guidelines.

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to the risk of individuals privacy being compromised but are available from the corresponding author on reasonable request.

Competing interests

Not applicable

Funding

This work was supported by the National Natural Science Foundation of China under Grant No. U20A20363 and Grant No. 81970776, the Natural Science Foundation of Heilongjiang Province, China under Grant No. LH2020H039

Author Contributions

Xuran Li and S Wang take charge of designing study and completing manuscript draft. Xuran Li, Rui Zhu and Jingrao Wang carry out the animal associated and histopathological experiments.

Hong Zhang coordinated financial support, and proof-read the manuscript.

Acknowledgements

Not applicable.

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Figures

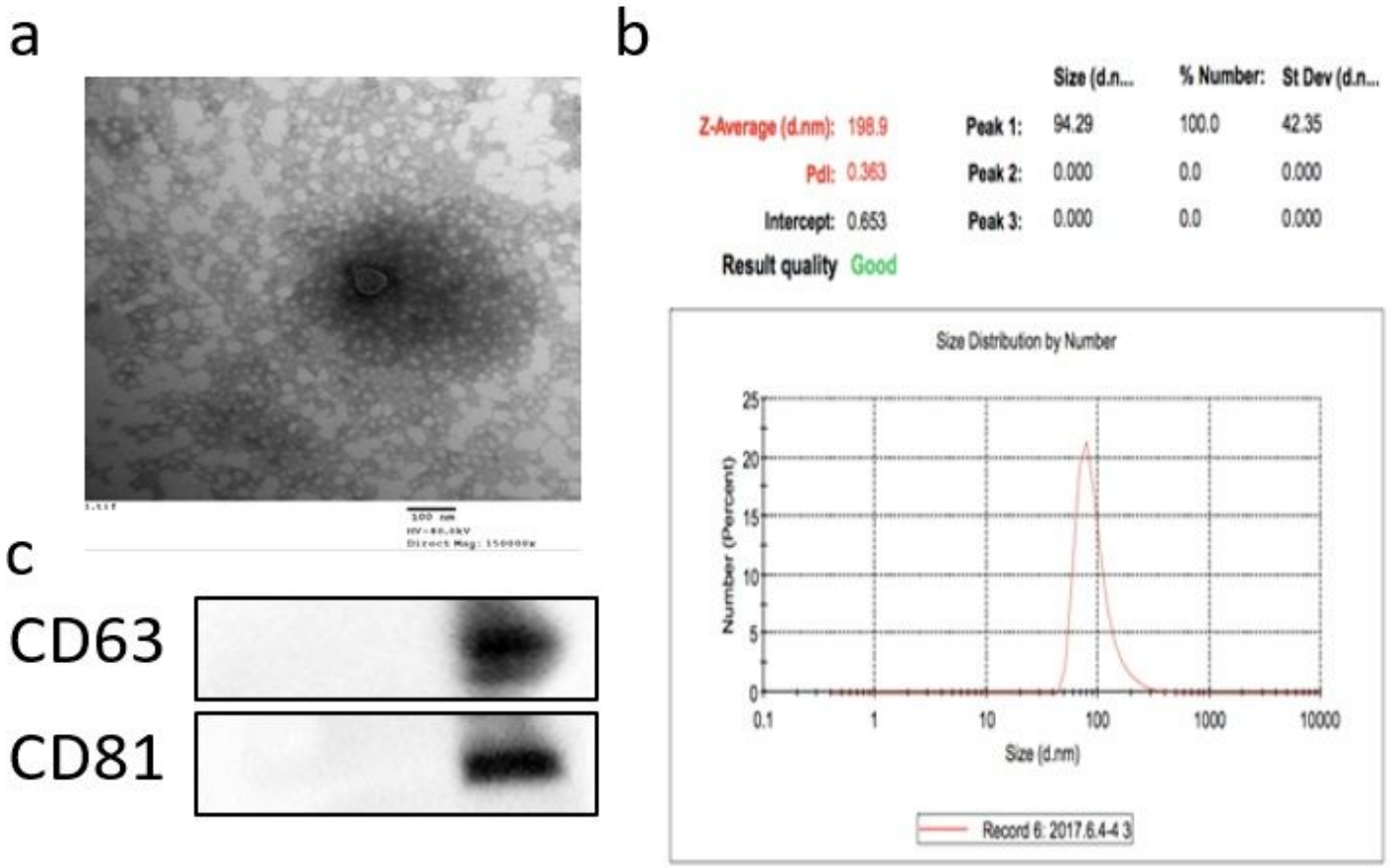

Figure 1

Characteristics of Exosomes

a, The morphologies of tear film exosomes under TEM with a diameter of near 100nm.

b, the diameter of Isolated exosomes is approximately 100 nm detected by DLS

c, WB indicated the known exosomal markers CD81 and CD63 was positive

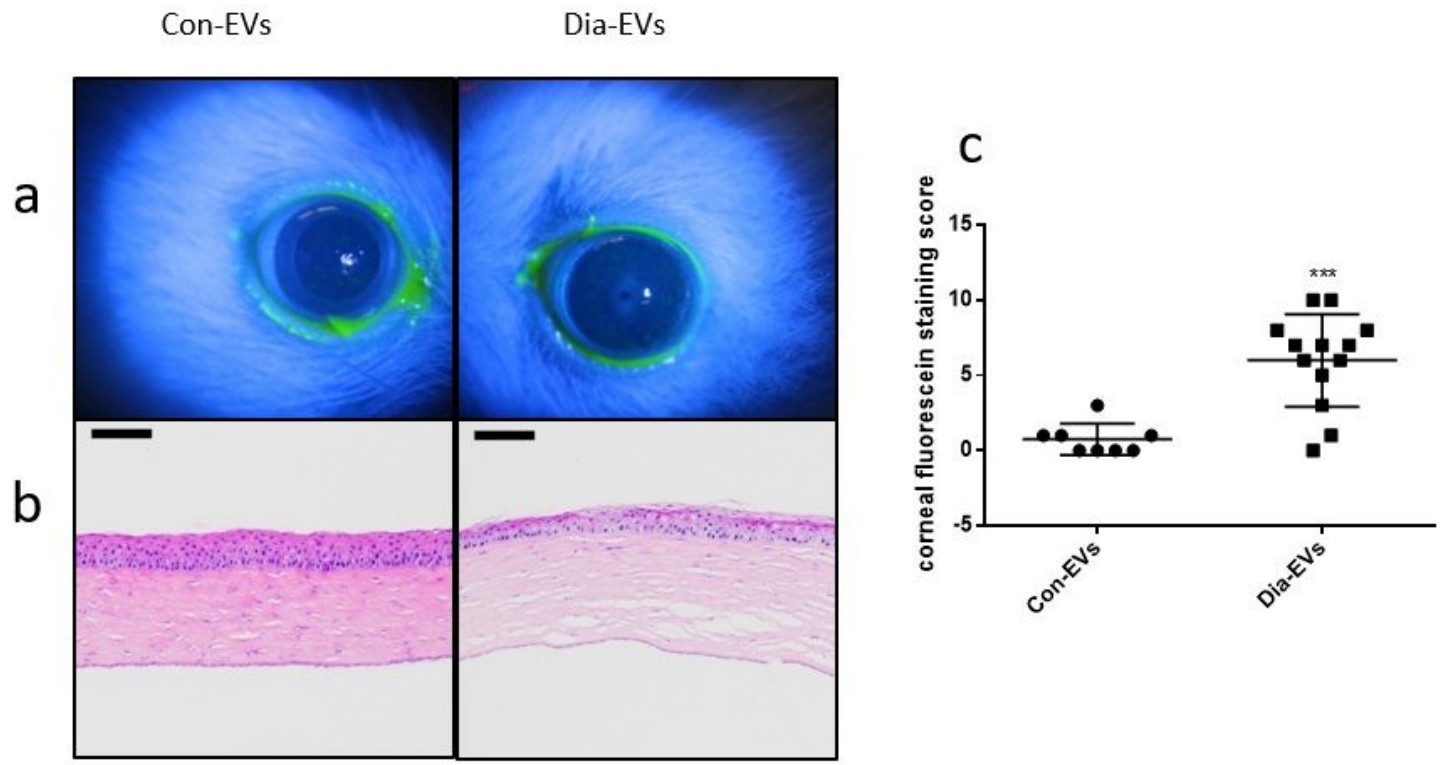

Figure 2

the cornea puncate defect contributed by Dia-EVs

a, fluorescein staining showed remianing sporadic positive staining of the corneal epithelium in Dia-EVs treated cornea, which increased, whereas there was little fluorescein staining in Con-EVs treated cornea.

b, H&E staining shows the disordered arrangement of the corneal epithelium. Scale bar represents 20 μm.

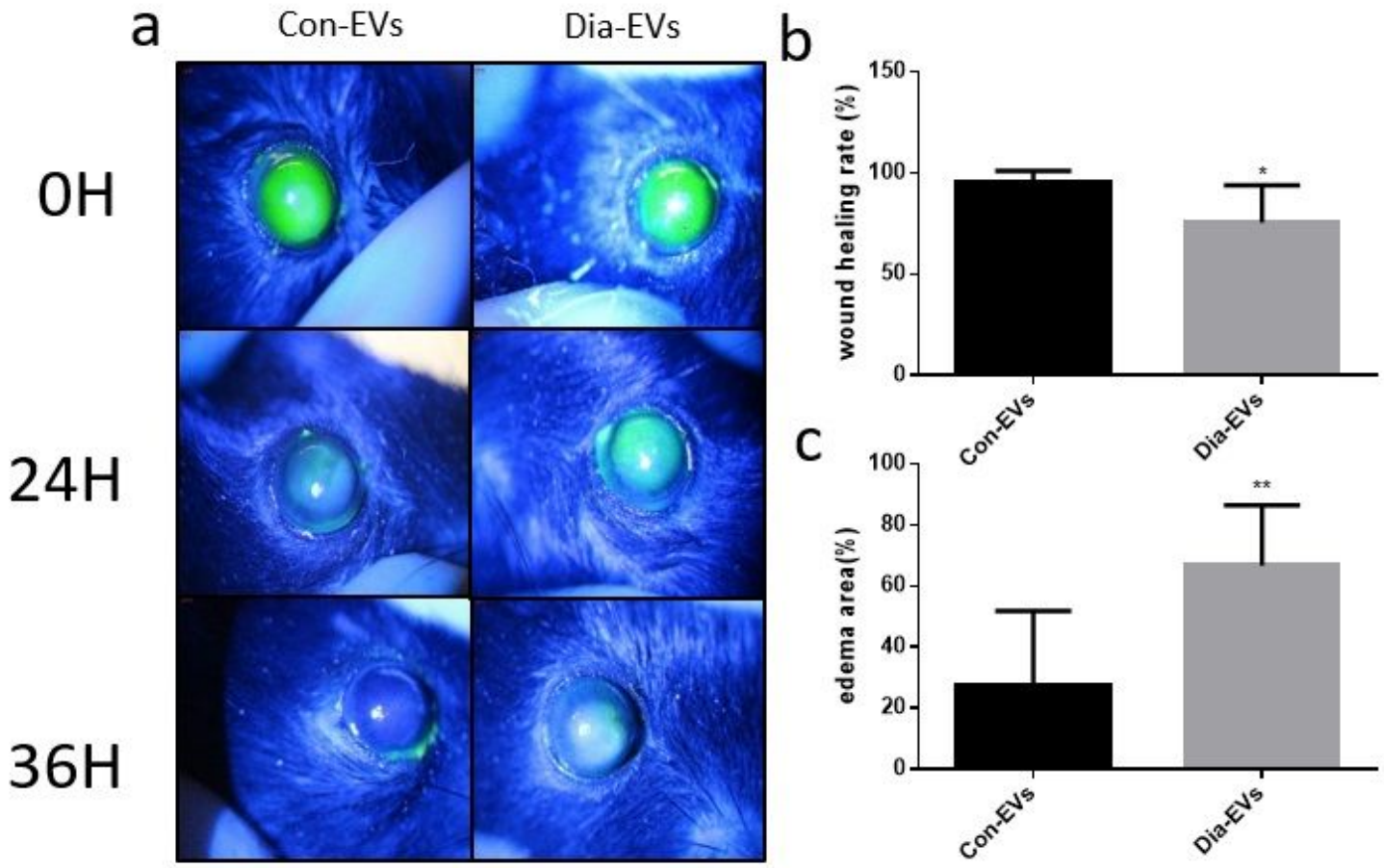

Figure 3

The recovery of corneal epithelial wound.

a, fluorescein staining showed the defect of corneal epithelium in Dia-EVs treatment group have a delayed recovery compared with corneas treated with Con-EVs, that almost completely healed in at 24 h

b, Dia-EVs treated cornea showed significant delayed corneal detumescence ($p < 0.05$*; $p < 0.001$***,).

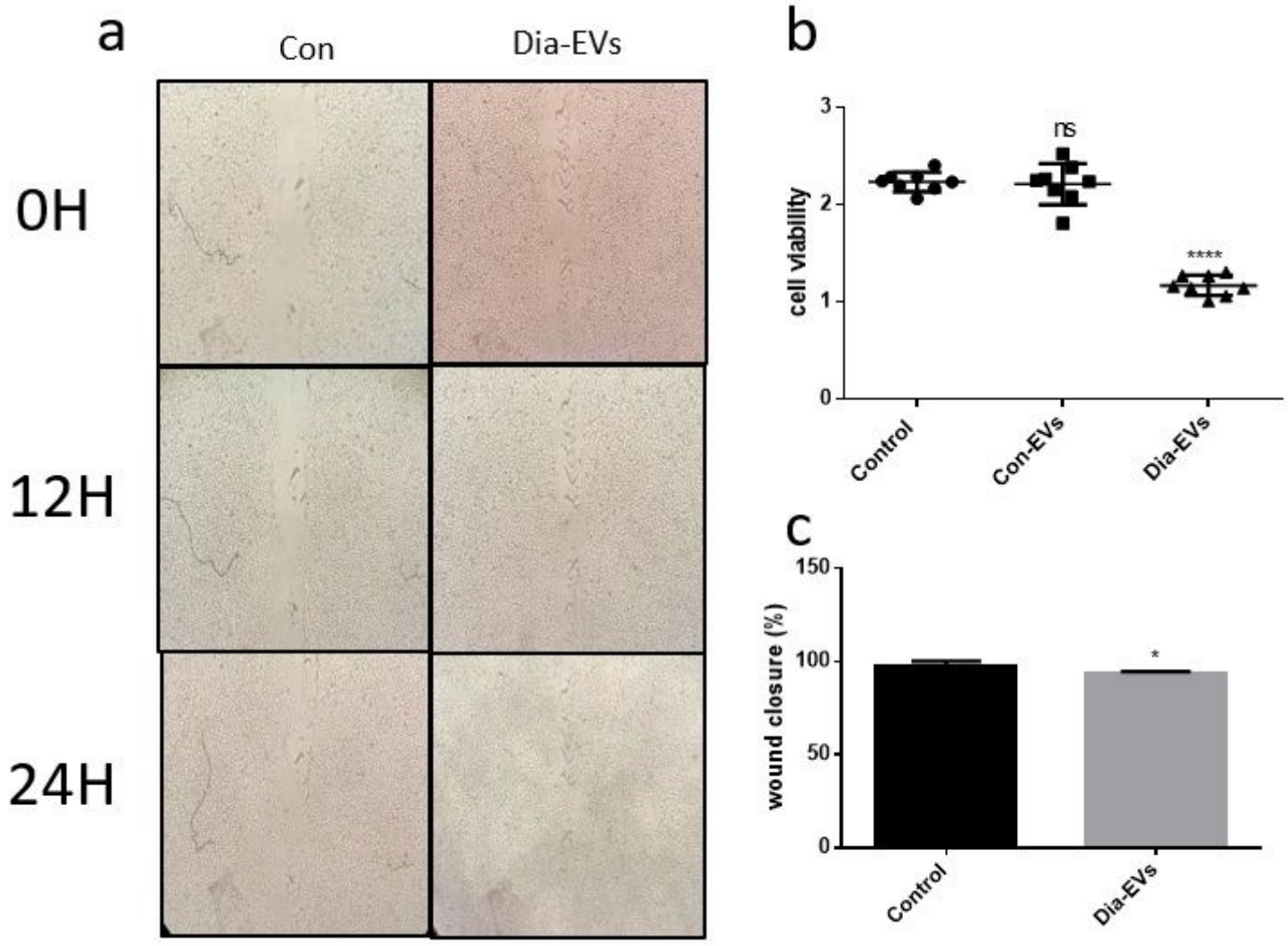

Figure 4

Dia-EVs decrease migration and proliferation of HCECs.

a, cell viability assay showed Con-EVs has no influence on proliferation of HCECs, while Dia-EVs treated cells has a mild decrease of viability at 24h compared to control.

b, Cell migration ability were significantly degenerated in Dia-EVs treated cells compared with the controls.

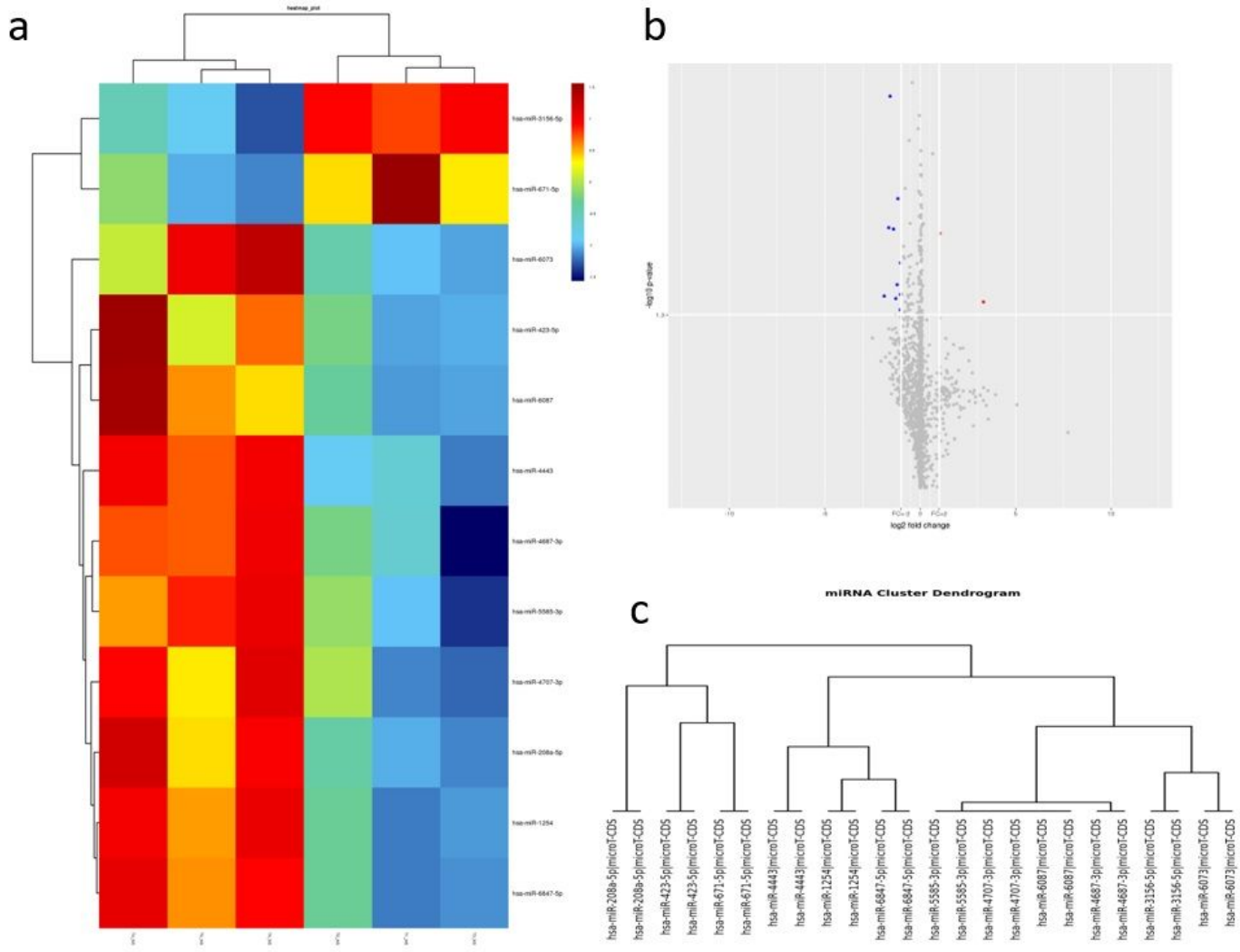

Figure 5

The graphical display of differential expression of miRNAs

a, The heat map shows the distinguishable miRNAs expression profile

b, Volcano plot shows the distribution of different expression base on up-regulated miRNAs (red spots) and down-regulated (green spots). X= log2 Fold Change; Y-=-log10 P value.

c, Hierarchical clustering analysis clustered miRNAs according similarity preferentially, could show the distances from miRNAs to miRNAs,

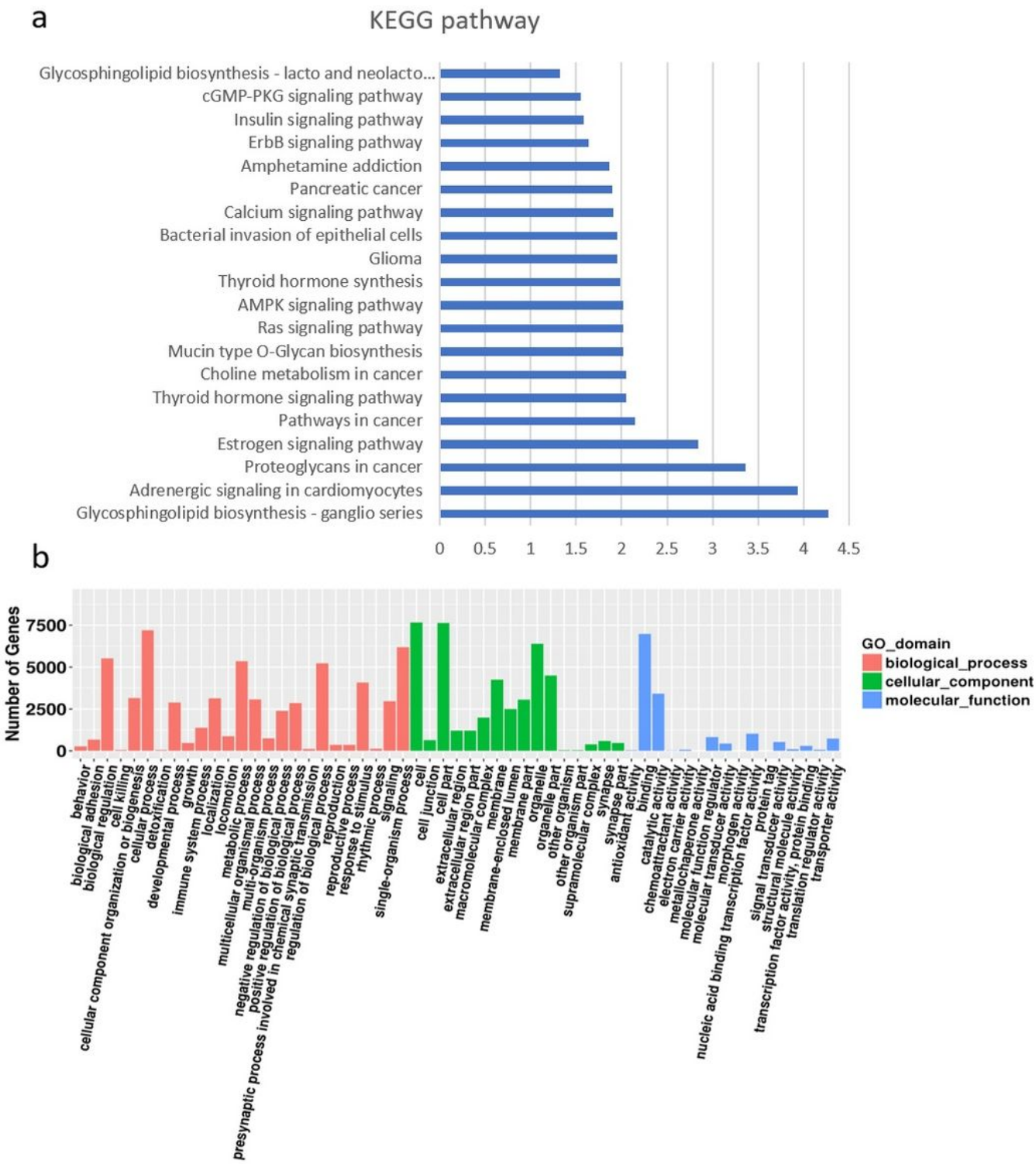

Figure 6

the KEGG and GO analysis of gene expression

a, KEGG pathway analysis

b, Enriched GO functions of differentially expressed genes