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Exosomes from adipose-derived stem cells restore fibroblast function and accelerate diabetic wound healing

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ABSTRACT

Background: Diabetes is common yet challenging chronic disease, that affects a wide range of people around the world. Complex cellular environments around diabetic wounds tend to damage the function of effector cells, including vascular endothelial cells (VECs), fibroblasts and epithelial cells. This study aims to analyze the differences between diabetic wounds and normal skin as well as whether adipose-derived stem cell (ADSC) exosome could promote healing of diabetic wound.
Methods: Human diabetic wounds and normal skin were collected and stained with HE, Masson, CD31 and 8-hydroxy-2 deoxyguanosine immunohistochemical staining. RNA-seq data were collected for further bioinformatics analysis. ADSC exosomes were isolated and identified by transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and western blotting. The effect of ADSC exosomes on diabetic wound healing was assessed on full thickness wounds in mice. To further verify the regulative impact of ADSCs exosomes in high glucose treated fibroblasts, we isolated fibroblasts from normal skin tissue and measured the cell viability, apoptosis rate, proliferation and migration of fibroblasts. In addition, collagen formation and fibrosis-related molecules were also detected. To further disclose the mechanism of ADSC exosomes on the function of high glucose treated fibroblasts, we detected the expression of apoptosis related molecules including BCL2, Bax, and cleaved caspase-3.
Results: Histological observation indicated that perilesional skin tissues from diabetic patients showed structural disorder, less collagen disposition and increased injury compared with normal skin. Bioinformatics analysis showed that the levels of inflammatory and collagen synthesis related molecules, as well as oxidative stress and apoptosis related molecules, were significantly changed. Furthermore, we found that ADSC exosomes could not only speed up diabetic wound healing, but could also improve healing quality. ADSC exosomes restored high glucose induced damage to cell viability, migration and proliferation activity, as well as fibrosis-related molecules such as SMA, collagen 1 and collagen 3. In addition, we verified that ADSC exosomes down-regulated high glucose induced increased apoptosis rate in fibroblast and the protein expression of Bax as well as cleaved caspases 3.
Conclusions: This study indicated that ADSC exosomes alleviated high glucose induced damage to fibroblasts and accelerate diabetic wound healing by inhibiting Bax/caspase 3.

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1. Introduction

Diabetes are common chronic diseases affecting a growing number of people around the world, among whom approximately 15% suffer from delayed wound healing and/or diabetic foot ulcers [1]. Poor blood supply combined with susceptibility to infection account for the complexity of diabetes wound management, making it the most common reason for amputation (14–24%) [2,3]. The main characteristics of diabetic wounds are difficulty in vascular reconstruction, peripheral neuropathy, and unmanageable infection [4]. In addition, hyperglycemia, hypoxia, and persistent inflammation tend to damage the function of effector cells of wound healing, including vascular endothelial cells, fibroblasts and epithelial cells [5,6]. Fibroblast is one of the most abundant cells in skin dermal tissue, playing a key role in wound healing [7]. Fibroblast is responsible for production as well as maintenance the extracellular matrix (ECM) by secreting and depositing collagen, while an unfavorable high-glucose environment induces excessive generation and accumulation of reactive oxygen species (ROS), which lead to the dysfunction of fibroblasts and increased apoptosis rate [8]. Impairment in fibroblast functions leads to restricted collagen synthesis and secretion, poor extracellular matrix formation and hence delayed wound healing [9]. Although a mass of efforts have been devoted to elucidating impairment wound healing of diabetes patients, the mechanism is still largely unknown. Hence, it is imperative to explore the mechanism of diabetic wounds and to develop novel therapeutic methods [10].

Adipose-derived stem cells (ADSC), one of the mesenchymal stem cells (MSCs), feature wide range of sources, easy isolation and amplification, as well as less immunogenicity [11]. It is well known that ADSC can promote wound healing via regulating cell function of a wide range of effector cells in wound healing [12]. Increasing evidence has revealed that therapeutic effect of ADSC depends on its paracrine factors, which are often transported with exosomes [12]. Exosome, one of extracellular vesicles with diameters ranging from 30 to 200 nm, is an important means of intercellular communication [13]. ADSC exosomes have been reported to be broadly used to protect against damage, inhibit inflammation and promote injured tissue repair and regeneration [14,15]. It is worth noting that ADSC exosomes could regulate the cell viability and function of effector cells in healing process, including VEC, fibroblast and epithelial cell [16–18]. More and more studies have reported that ADSC exosomes can promote wound healing in a wide variety of wounds, including chronic wounds and diabetic wounds [19–21]. But the underlying mechanism of therapeutic effect of ADSC exosomes still largely unknown, especially regulative effect on different cells in the process of wound healing.

ADSC exosome promotes neovascularization and formation of granulation tissue by inhibiting ROS and inflammatory cytokine accumulation in EPCs [22]. In addition, ADSC exosomes could inhibit inflammation in wounds by promoting promote macrophage M2 transdifferentiation [14,23]. Moreover, ADSC exosomes could significantly speed up the wound healing by enhancing the abilities of migration and proliferation of keratinocytes and fibroblasts [24]. In diabetic wounds, ADSC exosomes could enhance the oxidation resistance of endothelial cells and protect VECs from hyperglycemia induced oxidative stress injury [17]. It has been reported that ADSC-exosome, but not bone marrow mesenchymal stem cell (BMSC) exosome, promotes wound healing in a murine diabetic wounds model [25,26]. ADSC and BMSC exosomes were beneficial for effective cells involved in skin wound healing, such as fibroblasts, keratinocytes and ECs, but through different cellular processes. Another effective cell is fibroblast, which plays a role in ECM formation and closure of wound. However, the underlying mechanisms of the therapeutic effect of ADSC exosomes are still largely unknown and deserve further exploration. In particular, no single study has reported that ADSC exosomes could alleviate high glucose and ROS-induced fibroblast injury or its underlying mechanism.

In this study, we collected tissue samples of diabetic wounds and normal skin and analyzed their differences. We isolated ADSCs and extracted exosomes from the culture supernatant. We constructed diabetic wounds in a murine model and found that ADSC exosomes can significantly promote diabetic wounds and increase healing quality. Further mechanistic exploration firstly revealed that ADSC exosomes could restore hyperglycemia caused cell proliferation inhibition and dysfunction. In addition, ADSC exosomes improve collagen synthesis in hyperglycemia treated fibroblasts. Most importantly, ADSC exosomes could significantly change hyperglycemia induced fibroblast apoptosis via Bax/caspase-3. In conclusion, these results for the first time proved that ADSC exosome can promote diabetic wound healing via alleviate high glucose caused fibroblast ROS injury and apoptosis, which provide a solid theoretical basis for ADSC exosomes in diabetic wounds and provide new insight into the important role of ADSC exosomes in diabetic wounds.

2. Materials and methods

2.1. Ethical approval

Discarded skin tissue (dermis and adipose tissue) was obtained from voluntary patient undergoing abdominal operation in our department in the first affiliated Hospital of Fourth Military Medical University. All patients had a normal BMI, ranging from 20 to 23, and did not have other systemic diseases, such as hypertension, diabetes and hyperlipidemia. Before surgery, informed consents were signed by all patients and they all agreed to donate their tissue that was to be removed and discarded in their surgery. The design of this study was approved by the Ethics Committee of the first affiliated Hospital of Fourth Military Medical University.

2.2. Extraction and culture of fibroblasts

The extraction of fibroblasts was performed as previously reported [16]. Discarded normal skin tissue was collected and washed with sterile PBS containing 10 g/L penicillin–streptomycin (4 °C). The epidermis and subcutaneous tissue were separated and

removed, and the rest of the tissue was segmented and digested with 2 g/L collagenase type I (Sigma, Germany) at 37 °C for 0.5–1 h. The digested cells were passed through 100 μm and 70 μm cell filters and treated with DMEM containing 10% FBS to terminate digestion. Then, the cell was centrifuged at 300×*g* for 5 min, resuspended, cultured and passaged at 5% CO_2 and 37 °C. Cell experiments were conducted with 3rd to5th generation fibroblasts.

2.3. Extraction and identification of human adipose stem cells

Human subcutaneous adipose tissues were washed with sterile PBS containing 1 g/L penicillin–streptomycin (4 °C), wiped of the fascia and blood vessels, cut into small pieces, and then digested with 20 g/L collagenase type I. Then, the mixture was filtered by 100 μm cell screening, centrifuged at 300×*g* for 5 min and resuspended in culture medium. 3rd- to 5th-generation ADSC were incubated with antibodies (CD 73 CD 44, CD 29, CD 90, CD 34, and CD 45) and detected with flow cytometry. For osteogenesis experiments, ADSCs were seeded into six-well cell culture plate followed by osteogenic induction of differentiation at approximately 60–70% confluence. After treatment with osteogenic differentiation induction medium (Cyagen Bioscience, Inc.,China) for 3 weeks, the induced ADSC were fixed in 40 g/L paraformaldehyde and stained with Alizarin Red to view the induction results. For lipogenic differentiation, ADSC were seeded into six-well cell culture plate followed by lipogenic induction of differentiation at approximately 90–100% confluence. After treatment with lipogenic differentiation induction medium (Cyagen Bioscience, Inc., China) for 2 weeks, the ADSC were fixed in 4% PFM for more than 20 min then stained with Oil Red O to view the induction results.

2.4. Extraction and identification of exosomes

FBS were ultracentrifuged at 120,000×*g* for 16 h to remove cellular exosomes, and added to human ADSCs (third to fifth generation) cultured in 175 cm^2 cell culture flasks until 90% confluence. Conditioned culture medium collected from ADSC was then collected for exosome extraction. First, the samples were centrifuged at 300×*g* for 10 min to remove cells. Next, the samples were then centrifuged at 2,000×*g* to remove cell debris, followed by 10,000×*g* to remove apoptotic vesicles. Then, the samples were centrifuged at 100,000×*g* with a Ti70 rotor to collect particles, which were washed with PBS and settled with centrifugation at 100,000×*g* for 90 min again. All centrifugation steps were performed at 4 °C. The particles were resuspended in 100 μl of PBS and observed by transmission electron microscopy (TEM) and NTA. Simultaneously, western blotting was used to detect exosome markers TSG 101, CD 9 as well as CD 63. Exosomal protein concentrations were measured using the BCA Kit. Rest of the exosomes were stored at −80 °C.

2.5. Labeling of exosomes

Exosomes extracted from ADSC conditioned medium were filtered before the experiment and labeled with PKH26 to detect phagocytosis by fibroblasts. Briefly, 100 μl ADSC exosomes were incubated with PKH 26, then centrifuged at 100,000×*g* to remove uncombined dyes. Fibroblast was treated with ADSC exosomes for 24 h. After fixation in 4% PFM, the cells were stained with DAPI to show nuclei.

2.6. Cell experiment groups and treatment

For in vitro experiments, fibroblasts were divided into 3 groups: control, high glucose (34 mol glucose), and ADSC exosomes (34 mol glucose + 50 μg/mL ADSC exosomes). Twenty-four hours after different treatments, cells were collected for western blot and PCR analysis. In addition, to observe the proliferation and migration ability, differently treated fibroblasts were subjected to CCK-8, Transwell and scratch assays. ROS production in different groups was detected with ROS detection kit according to instructions of the manufacturer. DNA damage was observed via 8OHdG immunofluorescence staining.

2.7. Quantitative real-time polymerase chain reaction

Total RNA in both fibroblast sample and tissue sample were extracted with the TRIzol reagent. A total of 1000 ng RNA was reverse-transcribed to cDNA with the Prime Script™ RT kit (Takara, Japan) according to the manufacturer's instructions. Then, cDNA was amplified and detected by the Bio-Rad IQ5 real-time analysis system (Bio-Rad, Hercules, CA, USA) with specific primers. Relative expression levels were calculated using the 2-ΔΔCT method. Each reaction was performed in triplicate.

2.8. Western blotting

Fibroblast and skin tissue were washed and lysed with RIPA assay buffer with the help of ultrasonic. After lysis at 4 °C for 15 min, the cell and skin tissue lysis buffer were centrifuged at 12,000×*g* and denatured at 100 °C for 10 min. Fifty micrograms of protein was used in the blotting and then transferred to transfer membrane (Millipore, Billerica). Blocking with 5% nonfat milk, the membranes were probed with Col 1, Col 3, α-SMA, CD 9, CD 63, Bax, Bcl 2, caspase-3 and β-actin antibodies (1:1000). After incubation over night, the sample was incubated with HRP- secondary antibody. The proteins were detected by chemiluminescence, immunoblotting of membranes was probed with a FluorChem FC system (BioRad). β-Actin was used for the protein expression intensity.

2.9. Wound scratch test and Transwell assay

Fibroblasts were cultured in 6-well plates and stimulated with ADSC exosomes (50 μg/mL). Cells were scratched and treated with ADSC exosomes or PBS. After 0 h, 12 h and 24 h, distances of the scratch borders were observed and measured. Fibroblasts (2×10^4) were inoculated in chamber of transfer plate (Corning, NY) with a filter membrane pore size of 8 μm by adding 500 μL medium. One milliliter medium with ADSC exosomes or PBS was added for 24 h. Fibroblasts were then fixed and washed. Differently treated cells were stained with 5 g/L crystalline violet. Washing with PBS for 3 tines, migrating cells in different groups were observed.

2.10. Hematoxylin-eosin staining

Diabetic wound samples were fixed in 4 g/L paraformaldehyde, dehydrated, embedded, cut into 5-μm-thick sections and mounted. The sections were stained with hematoxylin–eosin (H&E) and Masson's trichrome and observed under an FSX100 microscope (Olympus).

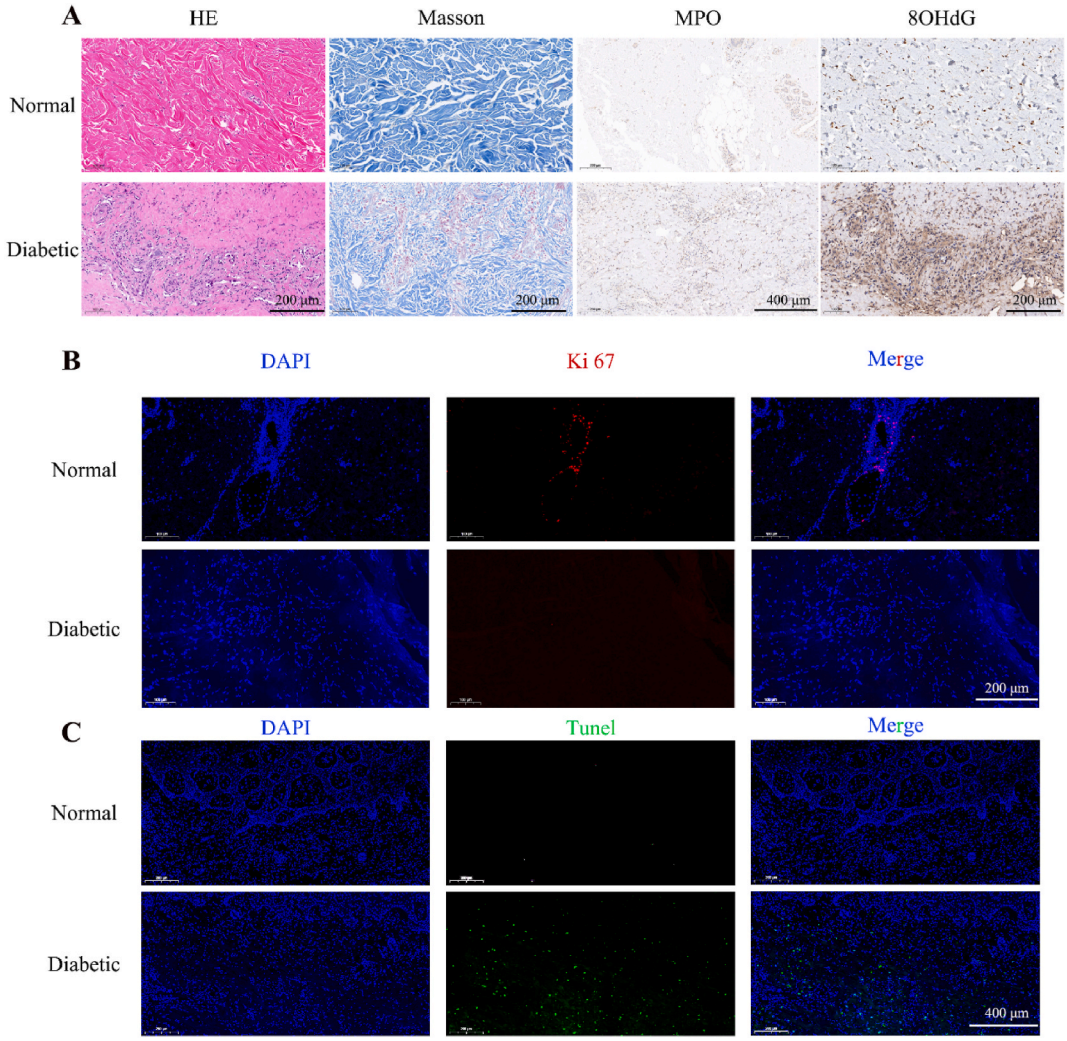

Fig. 1. Differences between diabetic wounds and normal skin. A. HE, Masson, MPO, and 8-OHdG staining results of diabetic wounds and normal skin. Scale bar: HE, Masson, and 8-OHdG 200 μM, MPO: 400 μM. B. Ki67 immunofluorescence staining results of diabetic wounds and normal skin, blue: DAPI, red: Ki67, scale bar: 200 μm. C. TUNEL immunofluorescence staining results of diabetic wounds and normal skin, blue: DAPI, green: TUNEL. Scale bar: 400 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.11. Immunofluorescence staining

Fibroblasts with different treatments were cultured in 6-well cell culture plates for 24 h. Fibroblasts were fixed in 4 g/L paraformaldehyde for 15 min at room temperature, washed three times with PBS, permeated with 1 g/L Triton X-100 in PBS for 20 min and blocked for 1 h. Different primary antibodies including α-SMA (1: 200, CST) Ki 67 (1: 200, CST) and 8 OHdG (1: 200, Abcam) were diluted in 20 g/L BSA and incubated overnight at 4 °C. Then, different treat cells and tissues were incubated with Fluorescent secondary antibody (1:100) for 1 h and counterstained with DAPI for further observation.

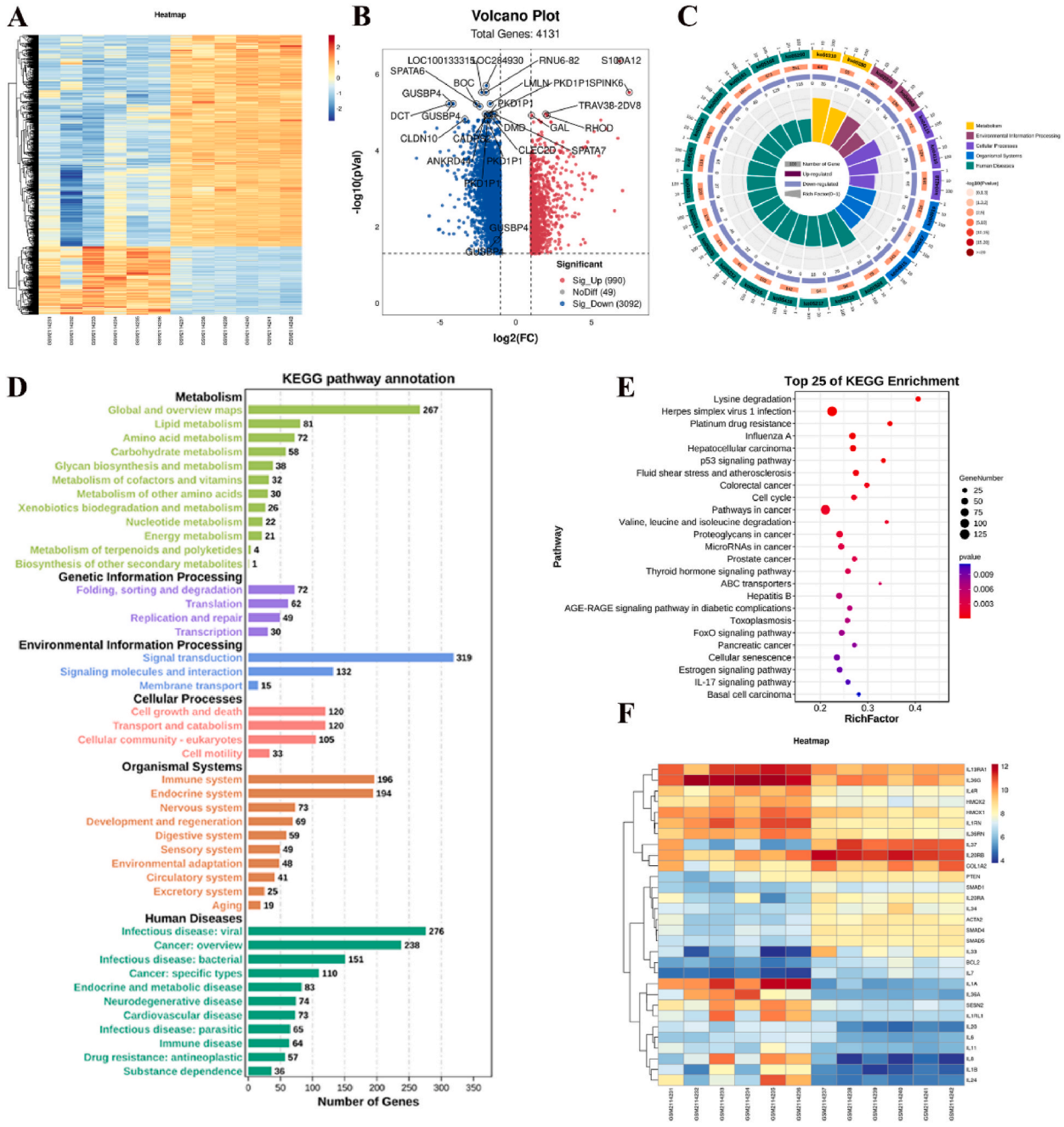

Fig. 2. Transcriptome sequencing revealed differences between diabetic wounds and normal skin. A Heatmap of the DEGs between diabetic wounds and normal skin. B. Volcano plot of the DEGs between diabetic wounds and normal skin. C. KEGG ontology enrichment of the DEGs between diabetic wounds and normal skin. D. KEGG pathway enrichment of the DEGs between diabetic wounds and normal skin. E. Top 25 KEGG pathway enrichment of the DEGs between diabetic wounds and normal skin. F. Heatmap showing the expression of inflammatory factors, collagen synthesis, apoptosis and oxidative stress.

2.12. Effect of ADSC exosomes on the wound model

To construct a diabetes model, 30 healthy BALB/c mice (7 weeks) were fed a high-fat and high-carbohydrate diet for 8 weeks, and 2.5 g/L STZ was injected from the 4th week (25 mg/kg). Blood glucose (BG) was measured via the tail vein, and all BG levels higher than 16.7 mmol/L were regarded as successful modeling. Mice were randomly divided into 3 groups (n = 8): PBS group (wild-type BALB/c), diabetes group, and diabetes + ADSC-Exo group (diabetic mice + 100 μg diluted in 100 μl of PBS). Mice were anesthetized with isoflurane, and a 1 × 1 cm^2 full-thickness skin defect was formed on the dorsal skin. Three days later, phosphate-buffered saline (PBS) or ADSC exosomes were administered subcutaneously into the wound perimeter for three consecutive days. Wounds were photographed on the 0, 3, 5, 7, 10 and 14 Days. Then, we sacrificed mice after 2 weeks and collected skin tissue for the histological analysis.

2.13. Statistical analysis

All data collected in this study were analyzed with GraphPad 8.0 software. All experiments were repeated randomly at least three times, and the data are presented as the mean ± SD. Student's *t*-test was used for comparisons between two groups, while one-way analysis of variance (ANOVA) was used for comparisons of more than two groups followed by Dunnett analysis between groups. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Differences between diabetic wounds and normal skin

To identify the difference between diabetic wounds and normal skin, we collected perilesional skin tissues from normoglycemic and

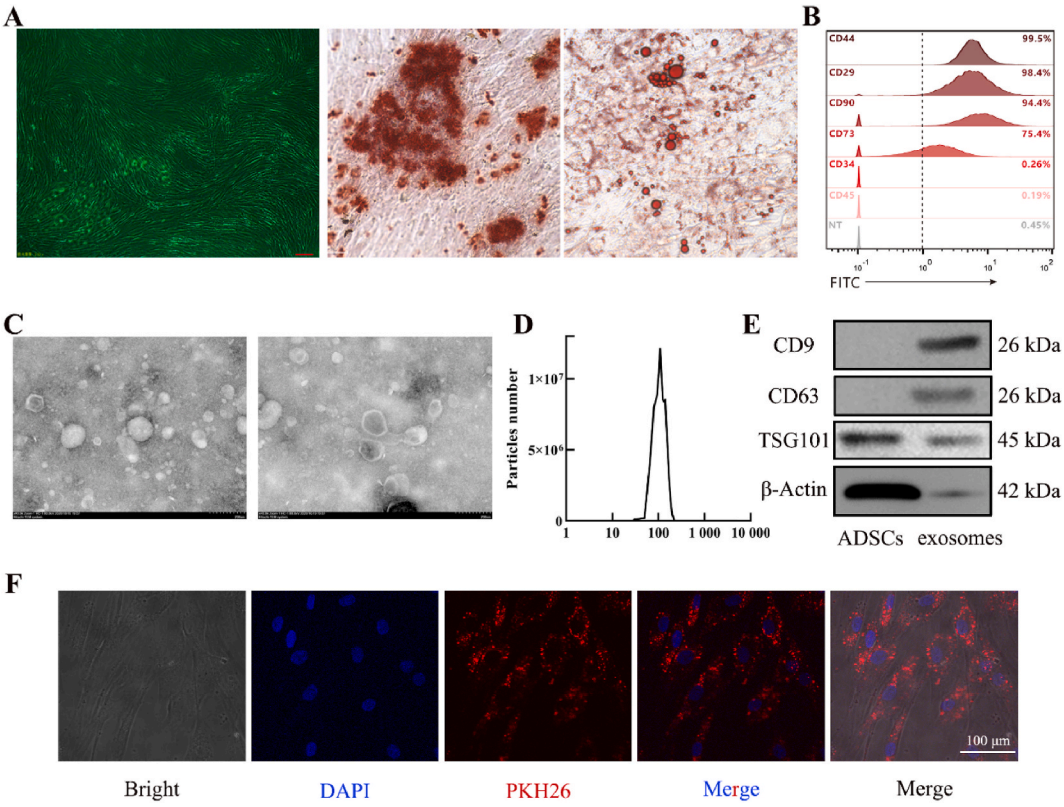

Fig. 3. Characterization of ADSCs and exosomes. A. ADSCs morphology observed by microscopy. Oil Red O staining and Alizarin Red S staining showing lipid droplets and calcium deposition after adipogenic and osteogenic induction, respectively. Scale bar: 25 μm. B. Flow cytometry showed that ADSC expresses CD 44 (99.5%), CD 29 (98.4%), CD 73 (75.4%), and CD 90 (94.4%) and negative for CD 34 (0.26%) and CD 45 (0.19%). C. TEM showing morphology of ADSC exosome. Scale bar: 200 nm. D. NTA showing diameter of ADSC exosomes (nm). E. western blotting showing expressions of CD 9, CD 63, TSG-101, and β-Actin in ADSC exosomes. F. Immunofluorescence staining results showing PKH26-labeled ADSC exosomes on fibroblasts; blue: DAPI, red: PKH26-labeled ADSC exosomes, scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

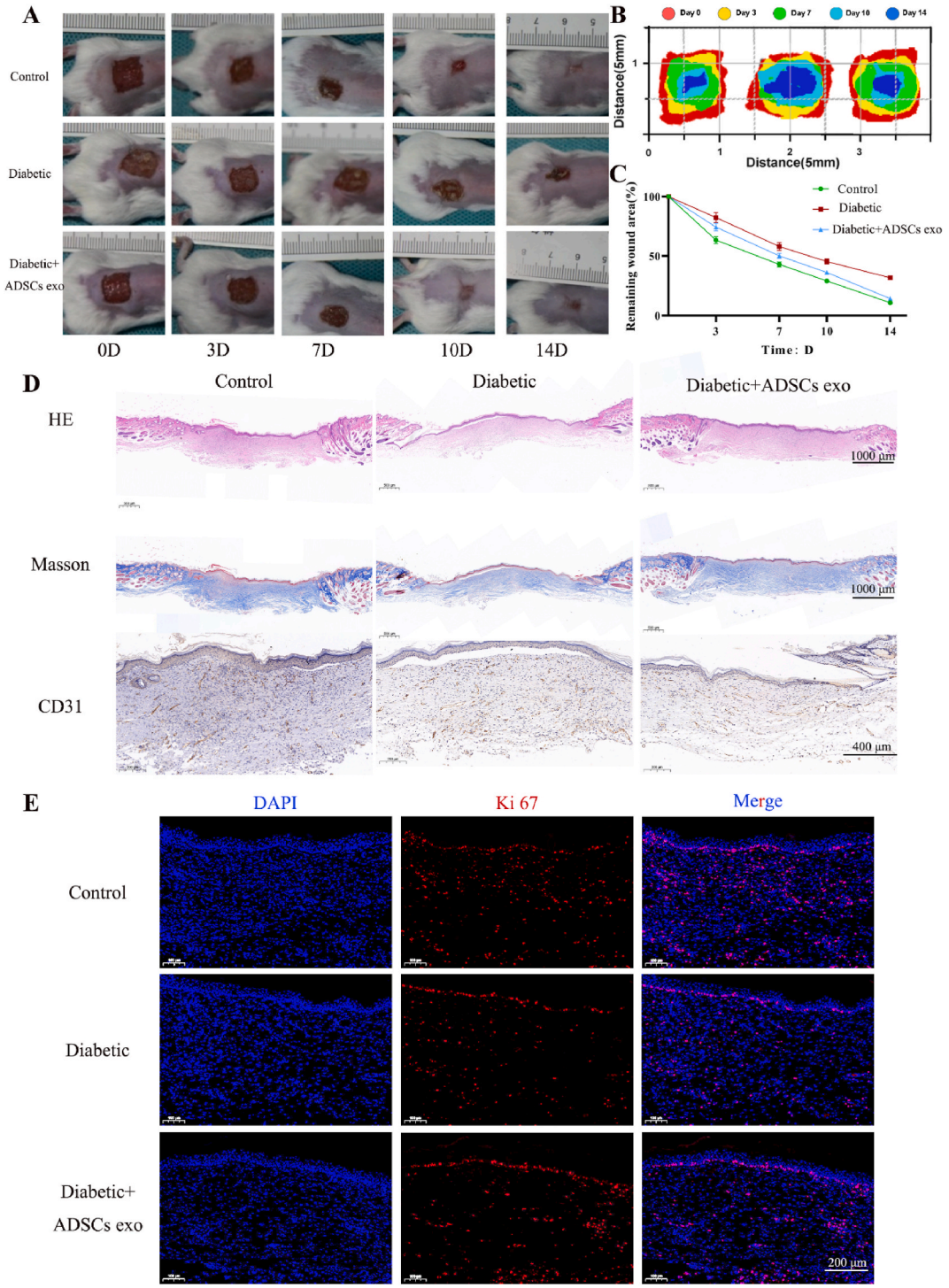

Fig. 4. ADSC exosomes promoted diabetic wound healing. A-C. Wound area of differently-treated mice (control, diabetes, and diabetes + ADSC exosomes) on Days 0, 3, 7, 10, and 14. D. HE, Masson and CD31 immunohistochemical staining results of wounds from different groups (control, diabetes, and diabetes + ADSC exosomes), scale bar: 100 μm, HE and Masson; 400 μm, CD31. E. Ki67 immunofluorescence staining results of wounds of the different groups (control, diabetes, and diabetes + ADSC exosomes), blue: DAPI, red: Ki67, scale bar: 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

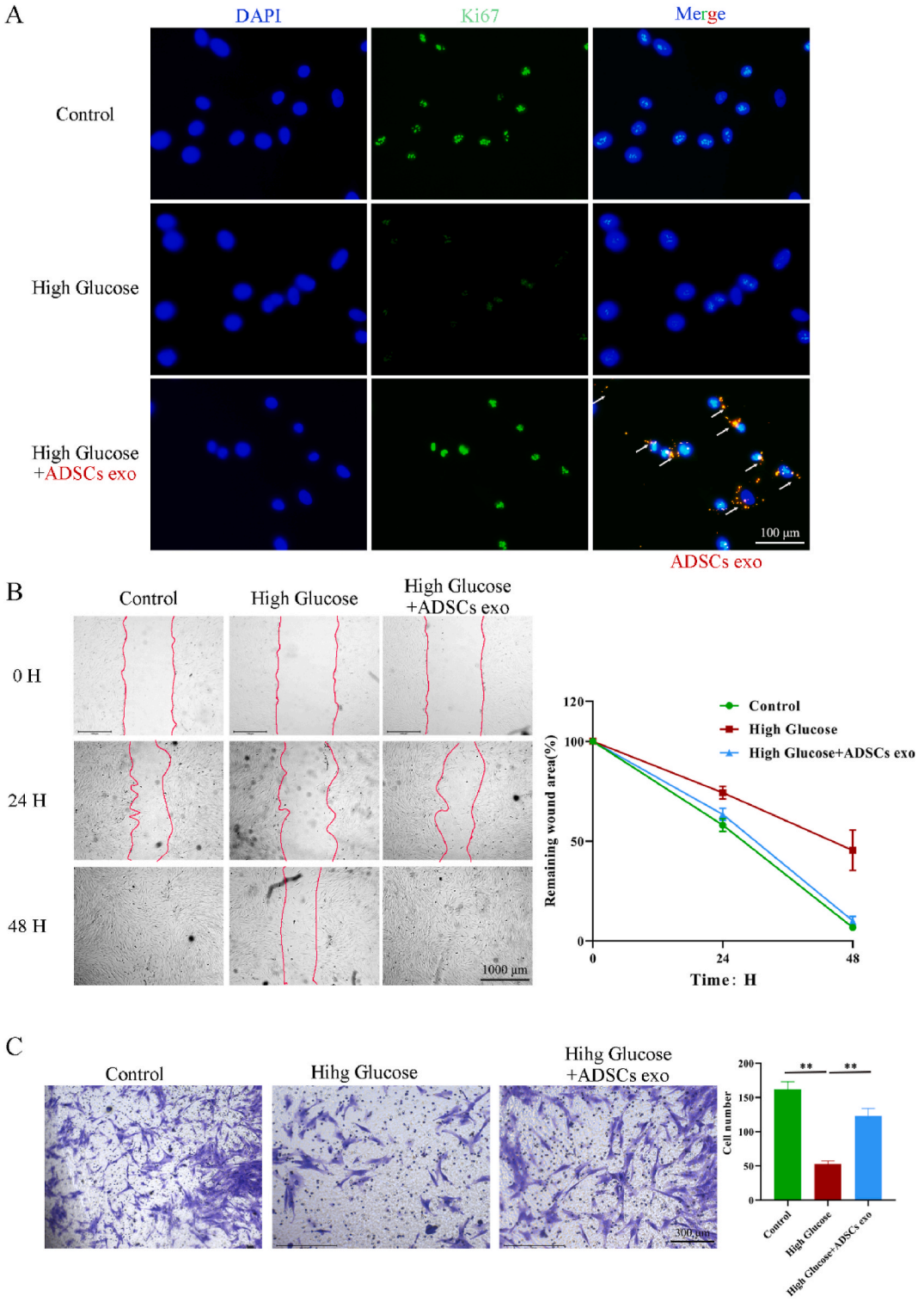

Fig. 5. ADSC exosomes improve proliferation and migration of high glucose cultured fibroblast. A. Ki 67 immunofluorescence staining results of NSFs treated with PBS, high glucose, or high glucose + ADSC exosomes. Blue: DAPI, green: Ki 67, red: PKH 26, scale bar: 100 μm. B. Migration evaluated by the scratch wound assays of NSFs treated with PBS, high glucose, or high glucose + ADSC exosomes; scale bar: 1000 μm. C. Migration evaluated by Transwell assays of NSFs treated with PBS, high glucose, or high glucose + ADSC exosomes, scale bar: 300 μm, * *: $P < 0.01$ (Dunnett test following one-way ANOVA). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

diabetic patients who were diagnosed with type 2 diabetes and diabetic wounds and underwent debridement in the Department of Burns and Cutaneous Surgery. To observe the histological characteristics, we performed HE staining and Masson staining. As is shown in Fig. 1A, tissues from diabetic wounds showed a loose dermis structure and more inflammatory cell infiltration, while the collagen distribution was sparser and collagenous fibers were shorter. 8-OHdG is one of the markers of DNA damage following oxidative stress injury, which is a common complication of delayed wound healing in diabetes. We conducted 8OHdG immunohistochemical staining to observe DNA damage in diabetic wounds and normal skin. The expressions of 8-OHdG and myeloperoxidase (MPO) in diabetic wounds were greatly higher than that in the control skin tissue. The Ki67 immunofluorescence staining results indicated that Ki67 positive cells in diabetic wounds was less than that in normal skin (Fig. 1B), while TUNEL-positive cells were more (Fig. 1C). These results indicated that perilesional skin tissues from nondiabetic patients showed structural disorder, less collagen disposition and increased injury compared with normal skin.

3.2. Transcriptome sequencing reveals differences between diabetic wound and normal skin

To further analyze the differences between diabetic wound and normal skin, we analyzed the RNA transcriptome sequencing results of diabetic wounds and normal skin reported by Sawaya AP et al. [27]. Fig. 2A showed that the expression of different genes in diabetic wounds and normal skin. As many as 990 genes were upregulated in hypertrophic scars, while 3092 were downregulated (Fig. 2B). Further bioinformatics analysis was conducted to analyze the differentially expressed genes. KEGG pathway enrichment indicated that the DEGs of two group are mostly enriched in global and overview maps of the metabolism category, signal transduction of environmental information processing category, immune and endocrine system of organismal system and infectious disease: viral and bacterial the of human disease category (Fig. 2C and D). As shown in Fig. 2E, the DEGs were enriched in the p53 signaling pathway, cell cycle, and microRNAs in cancer. Interestingly, we noticed that the expression of inflammatory and collagen synthesis-related molecules, as well as oxidative stress and apoptosis related molecules, was significantly changed, which might account for the delayed healing of diabetic wounds (Fig. 2F).

3.3. Characterization of adipose-derived stem cells and exosomes

Primary ADSCs isolated from adipose tissue showed a spindle shape under an inverted microscope (Fig. 3A). To verify the multiple differentiation potential of isolated primary ADSCs, adipogenic and osteogenic induction were conducted with P3 ADSC. Lipid droplets were observed by Oil Red O staining, and calcium deposition was observed by Alizarin Red S staining (Fig. 3A). Fig. 3B show that primary ADSC express CD 90, CD 44, CD 73 and CD 29, while CD 34 and CD 45 were almost not expressed. Then, we authenticated extracted ADSC exosomes with TEM, western blotting and NTA. The exosomes showed a cup-shaped typical morphology with a double-layer membrane structure under TEM (Fig. 3C). Moreover, Fig. 3D indicated that the particle diameters of ADSC exosomes ranged from 50 to 178 nm, with an average of 105.7 nm. Western blot analysis showed that molecular markers CD 63, CD 9 and TSG 101 were highly expressed in isolated ADSC exosome (Fig. 3E), while negatively expressed β-actin. As shown in Fig. 3F, PKH 26- ADSC exosomes could be taken by fibroblasts. These results showed that we isolated ADSC exosome and fibroblasts.

3.4. Adipose-derived stem cell exosomes promoted diabetic wound healing

To verify the effect of ADSC exosomes on process of wound healing, we established full-thickness wounds on diabetic mice backs and injected ADSC exosomes around the wounds every other day, while normal wild-type BALB/c mice were used as controls. As shown in Fig. 4A–C, compared with healthy BALB/c mice, diabetic mice showed delayed wound healing, while injection of ADSC exosomes significantly increased the healing of full-thickness wounds. On Days 3, 7, 10, and 14 post-wounding, the wound areas in the ADSC exosome group were smaller than those in the diabetic group. There were significant differences between the wound areas of the different groups on Days 3, 7, 10, and 14 (Fig. 4C). H&E and Masson's trichrome staining were carried out to evaluate the quality of wound healing. As Fig. 4D indicates, the wound areas in the ADSC exosome group were smaller than those in the control group, while cutaneous appendages in the ADSC exosome group were larger than those in the control group. The Masson results indicated that less collagen deposition and thinner, orderly arranged collagen structures were observed in the ADSC exosome group (Fig. 4D). More capillary formation in the ADSC exosome group was observed based on the CD31 immunohistochemical staining results (Fig. 4D). Moreover, the Ki67 immunofluorescence staining results indicated that ADSC exosomes could significantly increase the cell proliferation capacity around wounds (Fig. 4E). Therefore, the above results confirmed that ADSC exosomes could accelerate wound healing and improve the healing quality of diabetic wounds.

3.5. Adipose-derived stem cell exosomes improve the proliferation and migration of high glucose cultured fibroblasts

The above results indicated that wound healing was hindered in diabetic patients as well as in a mouse model and that fibroblasts were significantly influenced by high glucose concentrations, while ADSC exosomes could accelerate wound healing and enhance collagen deposition. It is reasonable to presume that ADSC exosomes could mitigate high-glucose induced fibroblast injury. To further verify our assumption, we extracted fibroblasts from normal healthy skin and treated them with high glucose and ADSC exosomes. As shown in Fig. S1, the CCK-8 results indicated that the cell viability of fibroblasts was attenuated when they were treated with high glucose. However, when fibroblasts were treated with ADSC exosomes, the viability of the high glucose induced fibroblasts injury was alleviated. As shown in Fig. 5A, intensity of Ki67 fluorescence in fibroblast was decreased when high glucose was added into fibroblast,

but increased when ADSC exosomes were used. Furthermore, both the Tran-swell and scratch assays indicated that high glucose lead to damage in the migration capability of fibroblasts, while ADSC exosomes restored it (Fig. 5B and C). Hence, these results revealed that high glucose leaded to damage of fibroblasts in the cell viability, proliferation, and migration, while ADS exosomes alleviated high glucose-induced damage.

3.6. Adipose-derived stem cell exosomes improve collagen synthesis in high glucose cultured fibroblasts

Fibroblast is one of the most abundant cells in dermal tissue, playing a key role in wound healing. Fibroblast is the major effector cell in wound healing, and is responsible for the production and maintenance of the ECM by secreted and deposited collagen. Hence, we further detected the expression of collagen-related molecules in differently treated fibroblasts. As shown in Fig. S2, the mRNA of Col 1, Col 3 and α-SMA was decreased after treatment with high glucose, which was significantly mitigated by ADSC exosomes. Moreover, the western blot results confirmed the same protein expression in differently treated fibroblasts (Fig. 6A). α-SMA is a marker of collagen synthesis and secretion by fibroblasts, so we further conducted immunofluorescence staining of αSMA. The expression of α-SMA in high glucose-treated fibroblasts was lower than control, while treatment with ADSC exosomes greatly increased the fluorescence intensity (Fig. 6B). These results indicated that ADSC exosomes restore high glucose induced collagen synthesis disorder in fibroblasts.

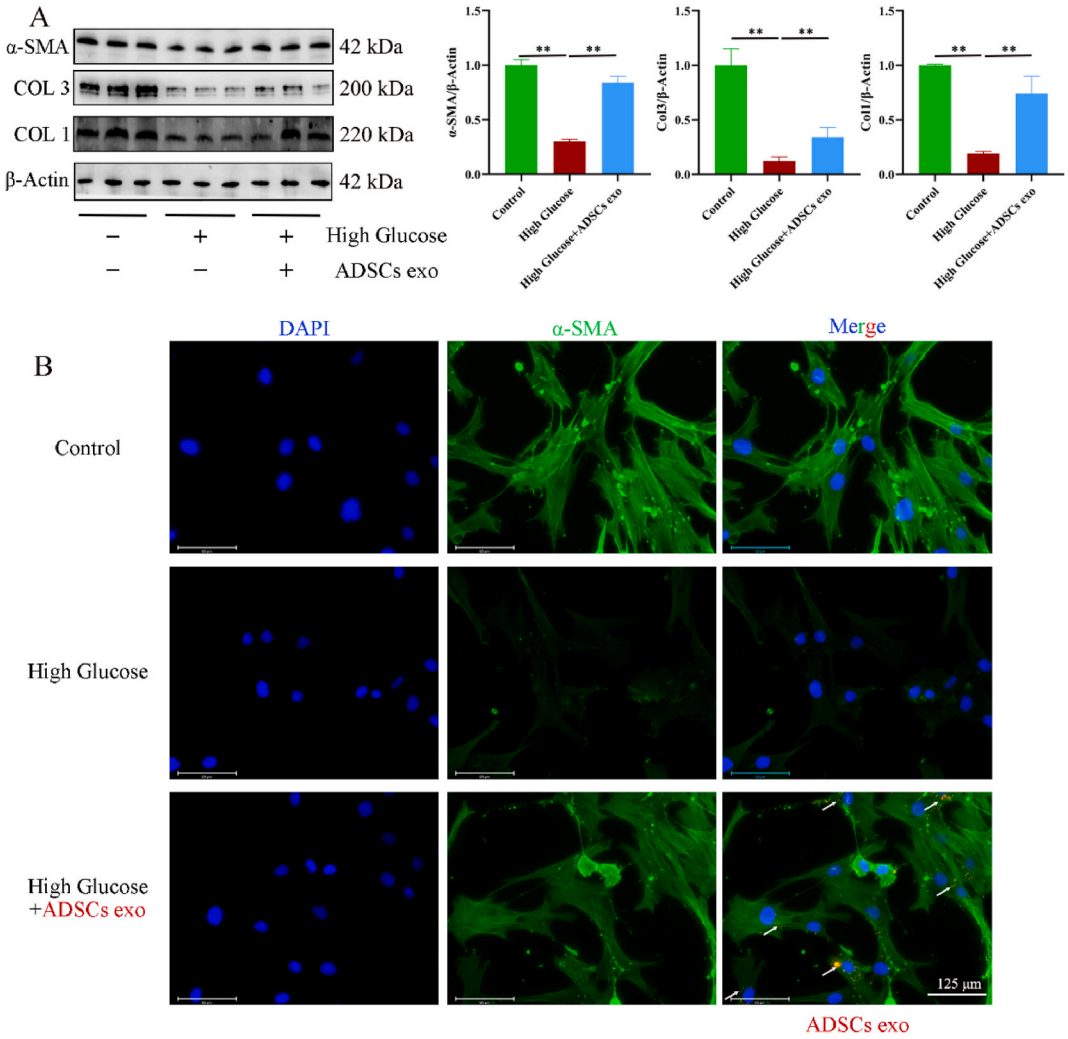

Fig. 6. ADSC exosomes improve the collagen synthesis of high glucose cultured fibroblasts A. Western blot results showing αSMA, COL1, and COL3 expression in NSFs treated with PBS, high glucose, or high glucose + ADSC exosomes, **: $P < 0.01$ (Dunnett test following one-way ANOVA). B. αSMA immunofluorescence staining results of NSFs treated with PBS, high glucose, or high glucose + ADSC exosomes, blue: DAPI, red: PKH26, green: αSMA, scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.7. Adipose-derived stem cell exosomes improve high-glucose induced oxidative stress injury

Previous studies revealed that high glucose treatment could induce oxidative stress injury in multiple cells in wounds, particularly vascular endothelial cells. We further detected the oxidative stress injury index of fibroblasts treated with high glucose and ADSC exosomes. As shown in Fig. 7A, when treated with high glucose, expression of ROS was more than control group. However, when treated with ADSC exosomes, the expression of ROS was significantly decreased. The flow cytometry results confirmed that ADSC exosomes could alleviate high glucose-induced fibroblast oxidative stress injury. 8OHdG is a marker of DNA damage. We further detected 8-OHdG expression in differently treated fibroblasts. The 8-OHdG immunofluorescence staining results indicated that high glucose treatment significantly increased the fluorescence intensity and positive rate of 8-OHdG, while ADSC exosomes alleviated the DNA damage of fibroblasts. Preceding results suggested that ADSC exosome alleviated oxidative stress injury in fibroblasts resulted from high glucose.

3.8. Adipose-derived stem cell exosomes improve high glucose induced fibroblast apoptosis

The above results showed that ADSC exosomes improve the cell function (proliferation, migration and collagen synthesis) as well as oxidative stress injury of high glucose cultured fibroblasts. To further illustrate the effect of ADSC exosomes on high apoptosis rate of glucose treated fibroblast, we detected apoptosis-related indicators (Bcl2, Bax and caspase-3). The results indicated that high glucose increased Bax and caspase-3 protein expression and decreased Bcl2 expression. However, ADSC exosomes significantly downregulated Bax and caspase-3 expression and upregulated Bcl2 (Fig. 8A). The flow cytometry indicated that the apoptosis rate in high glucose-cultured fibroblasts was higher than that of the control, while treatment with ADSC exosomes could decreased the apoptosis rate (Fig. 8B). These results suggested that ADSC exosomes significantly changed fibroblast apoptosis.

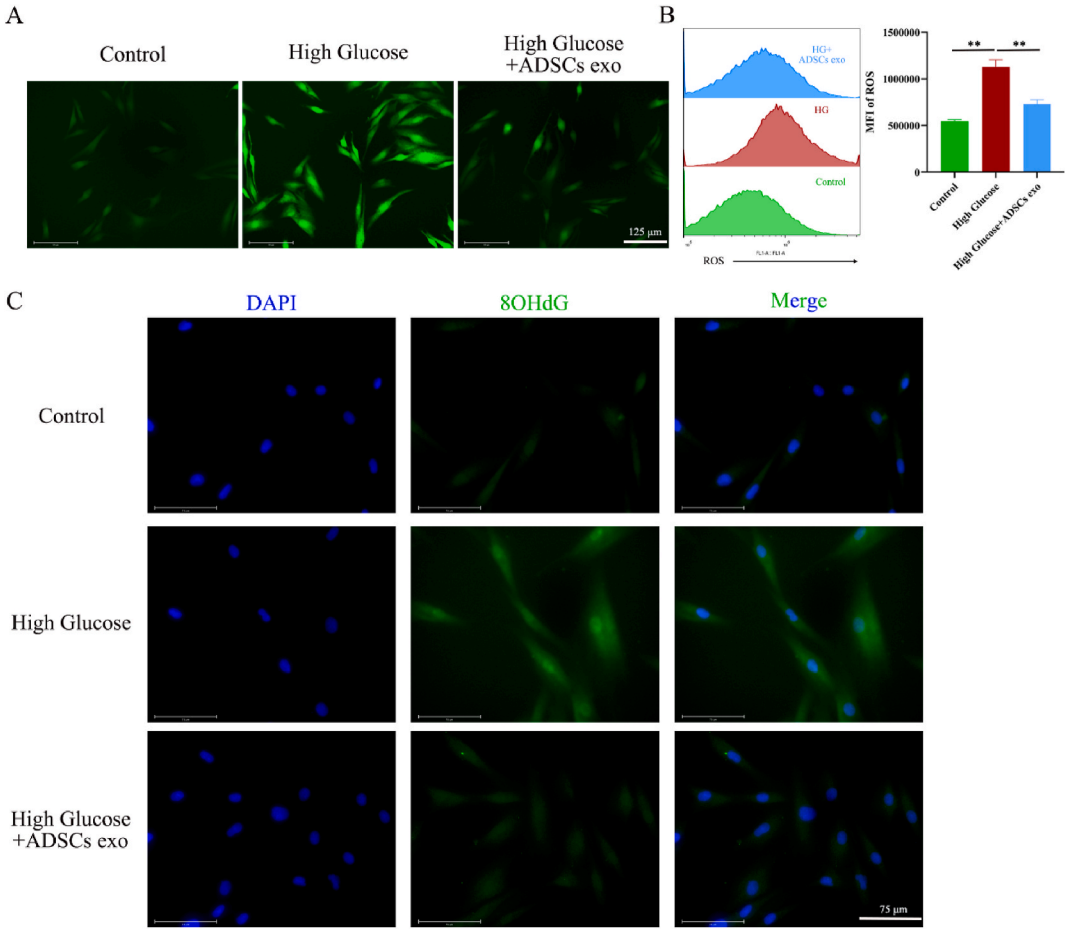

Fig. 7. Adipose-derived stem cell exosomes improve high glucose-induced oxidative stress injury. A. ROS immunofluorescence staining results of NSFs treated with PBS, high glucose, or high glucose + ADSC exosomes; scale bar: 125 μm. B. Flow cytometry and analysis results of ROS in NSFs treated with PBS, high glucose, or high glucose + ADSC exosomes, **: $P < 0.01$ (Dunnett test following one-way ANOVA). C. 8-OHdG immunofluorescence staining results of NSFs treated with PBS, high glucose, or high glucose + ADSC exosomes, blue: DAPI, green: 8-OHdG, scale bar: 75 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

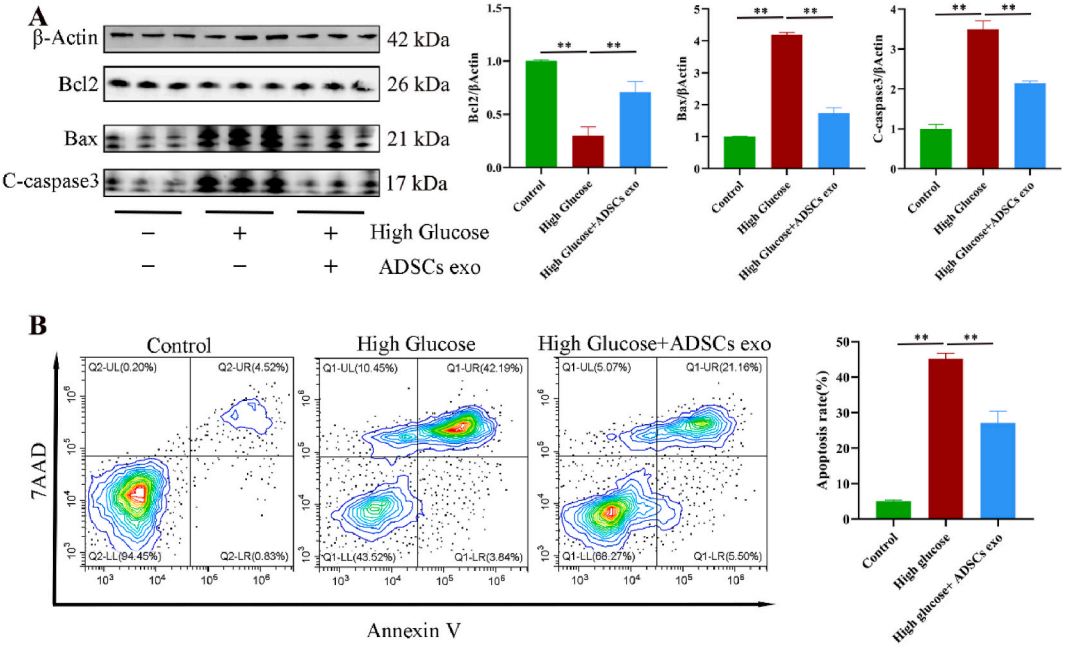

Fig. 8. ADSC exosomes improve high glucose induced fibroblast apoptosis. A. Western blot results showing Bcl2, Bax, and c-caspase-3 expression in NSFs treated with PBS, high glucose, or high glucose + ADSC exosomes, **: $P < 0.01$ (Dunnett test following one-way ANOVA). B. Flow cytometry results showing apoptosis rates of NSFs treated with PBS, high glucose, or high glucose + ADSC exosomes, **: $P < 0.01$ (Dunnett test following one-way ANOVA).

4. Discussion

Chronic diabetic wound is one of the public healthcare problems all over the world and are difficult to treat and cure, because an unfavorable high-glucose environment induces the accumulation of ROS [1]. In the process of wound healing, mutual collaboration of vascular endothelial cells, fibroblasts and epithelial cells leads to efficient wound healing [28]. Upon injury, cells around the wound are activated and recruited to the wound, where they initiate proliferation and fulfil their function. Vascular endothelial cells are responsible for angiogenesis, which delivers more oxygen and nutrients to the injured site, while fibroblasts are responsible for collagen synthesis and secretion, which constitute the ECM [7]. Normal cell function and an adequate cell number guarantee high-quality wound healing, while adverse factors often damage cell viability, thus leading to delayed wound healing [29]. In diabetic wounds, an unfavorable high-glucose environment induces the accumulation of ROS, leading to dysfunction and apoptosis of vascular endothelial cells, fibroblasts and epithelial cells. In this study, we collected perilesional skin tissues from normoglycemic and diabetic patients who were diagnosed with type 2 diabetes and diabetic wounds and analyzed the characteristics of diabetic wounds via histopathological examination and RNA-seq. The diabetic wounds presented a loose dermis structure and more inflammatory cell infiltration, as well as more severe DNA damage affecting proliferative activity. Through bioinformatics analysis of diabetic wounds and normal skin, we noticed that the expression of inflammatory- and collagen synthesis-related molecules and oxidative stress- and apoptosis-related molecules was significantly changed, which might account for the delayed healing of diabetic wounds.

To date, great attention has been given to the pathogenesis of and therapeutic methods for diabetes, and great progress has been achieved, although the healing is far from complete [3]. Many novel treatment strategies have been widely implemented [30]. Emerging studies have reported that MSC and their exosome is broadly applied in injury repair and regeneration owing to regulatory effect [31]. ADSC, a kind of MSC derived from adipose tissues, features abundant content, easy acquisition and convenient storage. ADSC exosomes can exert anti-inflammatory and antiaging effects, promoting damage repair and exerting immunomodulatory effects even in the absence of ADSCs [32]. ADSC exosome, one of the extracellular vesicles with a diameter at 30–150 nm, is a means of intercellular communication [33]. Almost all cells can secrete exosomes, which contain abundant components such as lipids, proteins and nucleic acids [13]. Once exosomes are taken up by other cells, their components exert corresponding regulatory effects depending on the source of the exosomes. Therefore, similar to ADSC, exosomes can protect tissues from certain wounding agents and speed up the regeneration of damaged tissue [34]. We extracted exosomes from ADSC, and authenticated them with NTA, TEM as well as western blotting. In addition, ADSC exosomes were used to verify their effect on diabetic wound healing. Unsurprisingly, ADSC exosomes significantly increased wound healing in diabetic mice. ADSC exosomes could not only increase new blood vessel formation, but could also promote collagen secretion and ECM formation, which is the foundation of wound healing.

Fibroblasts are important effective cells in wound healing, and are responsible for collagen secretion and ECM formation [35]. Upon skin injury, fibroblast is activated by injury stimulation or inflammation response and polarized to myofibroblast, with robust collagen synthesis activity and contractility [36]. During wound healing, fibroblasts mainly account for collagen secretion and ECM

synthesis. There is no doubt that more collagen synthesis is beneficial to wound healing, but uncontrolled collagen synthesis leads to HS [16]. However, when fibroblasts experience unfavorable factors, such as hypoxia, hyperglycemia, excessive inflammation and ROS, cell viability and collagen secretion and the synthesis activity of fibroblasts are significantly suppressed, which delays wound healing [27]. There is no doubt that an unfavorable environment in diabetic wounds influences the cell function of fibroblasts and resulted in delayed wound healing, while restoring cell function of fibroblasts accelerates wound healing of diabetic patients [9]. In this study, we found that high-glucose environment impaired viability, proliferation as well as migration of fibroblast while increasing the injury and apoptosis of fibroblast. Moreover, unfavorable high glucose levels induced ROS damage, led to DNA damage, and decreased collagen secretion and the synthesis activity of fibroblasts, which accounted for the delayed wound healing of diabetic wounds to a great extent. However, ADSC exosomes not only increased the cell viability, migration and proliferation of fibroblasts but also restored the collagen secretion and synthesis activity of fibroblasts. There are a large number of studies reporting that ADSC exosomes could promote wound healing, but few if any studies have reported that ADSC exosomes restored high glucose-induced fibroblast dysfunction. In addition, apoptosis-related analysis showed that ADSC exosomes could inhibit high glucose-induced fibroblast apoptosis by regulating the classic apoptosis pathway.

5. Limitation

This study has potential limitations. The effect estimates in the model are based on observational and interventional studies. RNA-seq data were reanalyzed distinction between diabetic wounds and healthy skin. However, RNA-seq can only display the overall genetic expression of the skin tissue and cannot reveal the injury and genetic expression of fibroblasts. Therefore, the best choice is 10× single-cell sequencing. However, limited by expenses and tissue samples, we were only able to use previously published data. In addition, in the mechanism research part, the experiment and design were superficial. It would be better to use genetically modified mice to clarify the mechanism. Therefore, further research should be devoted to mechanistic research, especially analysis of the exosome contents and regulatory effects.

6. Conclusion

In conclusion, in this study, we analyzed the differences between diabetic wounds and normal skin via histopathological examination. Besides, we analyzed the RNA-seq data of diabetic wounds and normal skin. In a diabetic wound model, we found that ADSC exosome accelerates the wound healing process of diabetic by improving angiogenesis, collagen deposition as well as ECM formation. In vitro experiments indicated that high glucose not only impaired the cell viability, migration and proliferation of fibroblasts but also decreased the collagen secretion and synthesis activity of fibroblasts. We further reported that ADSC exosomes could restore high glucose-induced fibroblast dysfunction by inhibiting high glucose-induced apoptosis. In brief, this study illustrated the mechanism underlying ADSCs accelerating diabetic wound healing and laid a foundation for the clinical application of ADSC exosomes for treating diabetic wounds.

Consent for publication

All authors approved the submission of the manuscript to this journal.

Ethics approval and consent to participate

All protocols used in the study involved human samples were approved by the First Affiliated Hospital of Air Force Medical University Ethics Committee.

Data availability statement

The data used to support the findings of this study are available from the corresponding author upon request. Data of RNAseq were downloaded from GEO database (GSE 134431).

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Relationships

There are no additional relationships to disclose.

Patents and intellectual property

There are no patents to disclose.

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CRediT authorship contribution statement

Chen Yang: Writing – original draft, Methodology. **Hao Zhang:** Writing – original draft, Validation, Methodology. **Chen Zeng:** Visualization, Data curation. **Chenyang Tian:** Investigation, Funding acquisition. **Wenjun Liu:** Visualization. **Yuxi Chen:** Visualization, Validation. **Meiqi Jia:** Investigation. **Ruizhi Wang:** Investigation. **Kejia Wang:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Yu Li:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e22802.

Abbreviations

ADSCs	adipose-derived stem cells
ROS	reactive oxygen species
3′UTR	3′untranslated region
α-SMA	α-smooth muscle actin
ECM	extracellular matrix
miR	microRNA
TEM	transmission electron microscopy
NTA	nanoparticle tracking analysis

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