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Basic Study

Exosomes from circ-Astn1-modified adipose-derived mesenchymal stem cells enhance wound healing through miR-138-5p/SIRT1/FOXO1 axis regulation

ADSCs exosome enhance wound healing

Zhi Wang, Cheng Feng, Hao Liu, Tian Meng, Weiqing Huang, Kexin Song, Youbin Wang

Abstract

BACKGROUND

Wound healing impairment is a dysfunction consequence induced by hyperglycemia and its effect on endothelial precursor cells (EPCs) in type 2 diabetes mellitus (T2DM). There is increasing evidence showing exosomes derived from adipose-derived mesenchymal stem cells (ADSCs) exhibit potential to improve endothelial cell function along with the wound-healing process. But the potential therapeutic mechanism of ADSCs exosome to wound healing in diabetic mice remains unclear.

AIM

METHODS

In this study, exosomes from ADSCs and fibroblasts were used for high-throughput RNA-Seq. ADSC-exosome-mediated healing of full-thickness skin wounds in diabetes mouse model was investigated. We utilized EPCs to investigate the therapeutic function of exosomes in cell damage and dysfunction caused by high glucose (HG). We utilized a luciferase reporter (LR) assay to detect interactions among circ-Astn1, sirtuin (SIRT)1 and microRNA (miRNA) (miR)-138-5p. We employed diabetic mice to verify circ-Astn1 therapeutic effect on exosome-mediated wound healing.

RESULTS

High-throughput RNA-Seq detection showed that circ-Astn1 expression was increased in ADSC exosomes compared with exosomes from fibroblasts. Exosomes contaning high circ-Astn1 concentration had enhanced therapeutic effect in restoring EPC function under HG conditions by promoting SIRT1 expression. circ-Astn1 expression enhanced SIRT1 expression through miR-138-5p adsorption, which was validated by LR assay along with bioinformatics analyses. Exosomes containing high concentrations of circ-Astn1 had better therapeutic effect on wound healing *in vivo* comparing to wild-type

(WT) ADSC exosomes. Immunofluorescence and immunohistochemistry investigations suggested that circ-Astn1 enhanced angiopoiesis through exosome treatment of wounded skin as well as suppressing apoptosis through promotion of SIRT1 and decreased FOXO1 expression.

CONCLUSION

In summary, we concluded that circ-Astn1 promoted ADSC-exosomes therapeutic effect and thus improved wound healing in diabetes *via* miR-138-5p absorption and SIRT1 upregulation. Based on our data, we advocate targeting the circ-Astn1/miR-138-5p/SIRT1 axis as potential therapeutic alternative regarding diabetic ulcers.

Key Words: ADSC; circ-Astn1; diabetic; exosomes; angiogenesis

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Core Tip: Circ-Astn1 promoted ADSC-exosomes therapeutic effect and thus improved wound healing in diabetes *via* miR-138-5p absorption and SIRT1 upregulation. Based on our data, we advocate targeting the circ-Astn1/miR-138-5p/SIRT1 axis as potential therapeutic alternative regarding diabetic ulcers.

INTRODUCTION

Diabetes influences 30 million children as well as adults in US, i.e., one out of every eleven people in America, which leads to $327 billion costs each year. So, It is important to develop a new method for diabetes treatment. Interventions that improve healing rates and decrease diabetic ulcer size could lower the infection incidence, amputation rate, and care cost [1]. Diabetic foot (DF) is a severe complication regarding T2D. DF infection (DFI) is main reason for DF development and deterioration, and controlling

infection functions importantly in disease treatment. Previous studies found that diabetes is associated with hyperglycemia, one of the most important causes of oxidative stress. Endogenous antioxidants are able to destroy the reactive species and create a balance between antioxidant and free radicals [2, 3]. The impaired function and senescence of endothelial progenitor cells (EPCs) and high glucose-induced ROS likely exacerbate DFs [4].

The accumulation evidence found that mesenchymal stem cell (MSC) transplantation can promote angiogenesis and accelerate diabetic wound healing [5, 6]. Therapy using ADSCs is developing into new therapeutic option to improve diabetic wound healing [7], and autologous stem cell transplantation reduces the cost of drug development, which in turn reduces financial costs. But the mechanism is not clear.

Stem cells live in niches, complicated microenvironments which exert important functions in directing the division, differentiation and activity of stem cells. However the direction of differentiation is affected by hypoxia, cytokines, trophic factors, chemical and pharmacological agents, and physical factors [8]. Considering the safety of *in vivo* transplantation, some investigations have suggested that exosomes (Exos) from ADSCs play an similar functional role to ADSCs in promote diabetic wound healing. Exos are tiny endosomal membrane-bound vesicles 50–200 nm in length and having a variety of contents including protein and nucleic acids which vary with their cell or tissue origin. They play their full role through fusing with selected cells and releasing their cargo that could contain bioactive molecules including lipids, proteins, non-coding-RNA (ncRNA) [9-11] and mRNAs. Previous studies have found that exosomes can regulate the epithelial-mesenchymal transition and the progress in different cancer [12, 13]. Exosome secreted from ADSCs attenuates diabetic nephropathy by promoting autophagy flux and inhibiting apoptosis in podocyte [14]. Exosomes from Nrf2 overexpressing ADSCs accelerate cutaneous wound healing by promoting vascularization in a diabetic foot ulcer [4]. Exosomes from linc00511-overexpressing ADSCs accelerates angiogenesis in diabetic foot ulcers healing by suppressing PAQR3-

induced Twist1 degradation [15]. However it remains largely unknown whether Exos from ncRNA-modified ADSCs can improve wound healing.

The ncRNAs include circular RNA (circRNA), long non-coding RNA (lncRNA) and microRNA (miRNA). circRNA activity is indispensable during regulation of gene expression, demonstrating that circRNAs function not only as candidate therapeutic agents but also as diagnostic markers. circRNA 5′ and 3′ extremities are linked to form an integrated circular structure which makes circRNAs more resistant to RNA exonuclease degradation as well as more stable than linear RNAs [16, 17]. A previous study found that circRNAs possess activity and potential clinical benefits in skin wound healing [18].

To identify relevant circRNAs as therapeutic targets, we used high throughput sequencing detection to identify the function of mmu_circ_0000101 (circ-Astn1), which acts as the key factor in delivery by ADSC Exos. Exos from circ-Astn1-modified ADSCs improve wound repair in diabetic rats through miR-138-5p/SIRT1 pathway regulation. The present study verified the effect of treatment with Exos from circ-Astn1-overexpressing ADSCs on high glucose (HG)-induced EPC dysfunction. The abundance and the simple methods of sampling of ADSCs-Exos make it safer against trauma and other adverse reactions.

MATERIALS AND METHODS

Ethics statement

Animal Care and Use Committee of Peking Union Medical College Hospital approved the investigation protocol (No: XHDW-2020-01). We carried out all postoperative animal care along with surgical interventions following NIH Guide for Care and Use of Laboratory Animals. All surgery and euthanasia were performed under sodium pentobarbital anesthesia (30 mg/kg) by intraperitoneal injection, and all efforts were made to minimize suffering.

High-throughput and strand-specific RNA-Seq library construction

Total RNA from ADSC and fibroblast Exos was isolated through TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Our team prepared around 3 μg total RNA per sample using a VAHTS Total RNA-seq (H/M/R) Library Prep Kit from Illumina (Vazyme Biotech Co., Ltd, Nanjing, China) to isolate the ribosomal RNA and remove other RNAs like ncRNA and mRNA. Our team then performed RNA purification using RNase R (Epicenter, 40 U, 37°C for 3 h) followed by TRIzol. An RNA-seq library was prepared through a KAPA Stranded RNA-Seq Library Prep Kit (Roche, Basel, Switzerland) and expose them in order following extensive codifying with Illumina HiSeq 4000 from Aksomics, Inc. (Shanghai, China).

Cell treatment

To detect endothelial precursor cell (EPC) dysfunction as well as apoptosis, we cultivated EPCs at 37°C with 5% CO_2 in EPC medium (Gibco, Carlsbad, CA, USA) and processed after 1 d using 5.5 or 30 mmol/L glucose. We also gathered EPCs for detection of apoptosis as well as to test their response to Exo therapy. In order to study the protective function of Exos on EPCs, we added 100 μg/mL Exos to cultures following 80% EPC fusion to evaluate the protective function against damage caused by prior HG treatment with various glucose concentrations.

ADSCs isolation and identification

We isolated ADSCs from adipose tissue following previous studies [4]. We observed no uninduced differentiation in cultural expansion. We induced osteogenic differentiation *via* 3-week culture of ADSCs in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.1 μM dexamethasone, 50 μM ascorbate-2-phosphate and 10 mmol/L β-glycerophosphate. We induced adipogenic differentiation through culturing ADSCs for 2 w in DMEM supplemented with FBS of 10%, 10 μM insulin, 0.5 mmol/L isobutylmethylxanthine, 200 μM indomethacin and 1 μM dexamethasone. We also investigated the osteogenic or adipogenic differentiation of ADSCs through Oil-Red O staining and alkaline phosphatase.

Identification and isolation ADSC-derived Exos

We isolated ADSC-derived Exos when cells reached eight to ninety percent confluence. Our team rinsed ADSCs from various groups with PBS, then cultured them in FBS-free endothelial cell growth medium (EGM)-2MV, which were supplemented with 1× serum replacement solution (PeproTech, Rocky Hill, NJ, USA) for another 2 d. Then our team collected conditioned culture medium and centrifuged them at 300 × *g* for 10 min to erase cells and at 2,000 × *g* for another ten minutes to erase apoptotic cells and cellular debris. Following centrifugation at 10,000 × *g* for 30 min, we filtered supernatant *via* 0.22 μm filter (Millipore, Billerica, MA, USA) then transferred 15 mL supernatant to Amicon Ultra-15 Centrifugal Filter Unit (100 kDa) and centrifuged it at 4,000 × *g* to concentrate to ~1 mL. Our team then washed ultrafiltration unit two times with PBS, centrifuged them again at 100,000 × *g* and aspirated supernatant. All processes were conducted at 4°C and we resuspended Exo pellets obtained in 500 μL PBS. Finally, the Exo protein content was evaluated *via* Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). We stored Exos at -80°C until we performed experiments and identified Exos by western blotting as well as by transmission electron microscopy.

Diabetic wound induction

We utilized Balb/c mice and induced diabetes through single intraperitoneal injection of 60 mg/kg streptozotocin (STZ) dissolved in 0.1 M citrate buffer (pH 4.5). At 3 d following STZ administration, we confirmed diabetes development through measuring fasting blood glucose levels in blood samples obtained from tail vein. We considered a mouse with fasting blood glucose levels > 250 mg/dL diabetic, which we maintained for 1 m and employed for subsequent analyses of posterior blood glucose stabilization. Following diabetes validation, we anesthetized mice through intramuscular injection with ketamine hydrochloride and xylazine cocktail at 80 and 10 mg/kg. Once anesthesia was established, hair was shaved from the dorsal leg area and the region was sterilized using povidone iodine solution. Sterile biopsy punch was used to generate full-thickness 4 mm excisional wound. We then allocated mice randomly to subcutaneous injection with 100 μL PBS containing 200 μg ADSC Exos or equivalent

amount of PBS without Exos at 4 sites near the wound (25 μL/site). We euthanized mice after 0.5 mm and harvested skin specimens for histopathological validation.

RNA overexpression or interference

RNA overexpression or interference was induced by miR-138-5p mimics or inhibitor transfections, circ-Astn1 and SIRT1 overexpression vector, siRNA againse circ-Astn1 (si-circ-Astn1) obtained from RiboBio (Guangzhou, China). Our team performed transfection *via* Lipofectamine 2000 (Thermo Fisher Scientific) following a method previously described [19].

Quantitative real-time polymerase chain reaction (RT-qPCR)

We isolated total RNA from skin tissue or cells from wound through a TRIzol reagent kit. Our team synthesized cDNA to amplify with TaqMan miRNA Reverse Transcription Kit. Our team then performed qPCR with TaqMan Human miRNA Assay Kit, utilizing $2^{-\Delta\Delta CT}$ approach to detect fold changes with respect to expression. We used *U6* and *GAPDH* as internal references. Primers utilized were: circ-Astn1, F: 5′-CTGGACCCTTGTGAACACCAATG-3′, R, 5′-GGATCATCACCAGGCACAAGATG-3′; FOXO1, F: 5′-AAGGCCATCGAGAGCTCAGC-3′, R, 5′-GATTTTCCGCTCTTGCCTCC-3′; miR-138-5p, F: 5′-GCTGGTGTTGTGAATCAG-3′, R: 5′-GAACATGTCTGCGTATCTC-3;
U6, F: 5′-AGTAAGCCCTTGCTGTCAGTG-3′, R: 5′-CCTGGGTCTGATAATGCTGGG-3′; GAPDH: F: 5′-GTCTCCTCTGACTTCAACAGCG-3′ , R: 5′-ACCACCCTGTTGCTGTAGCCAA-3′, and were designed by Gene Pharma (Shanghai, China).

Apoptosis detections

To assess apoptosis, we collected cells into centrifuge tubes and centrifuged them at 1000 rpm for five minutes. We resuspended cells in PBS at 4°C and we removed supernatant following centrifugation. We resuspended cell pellet at 1–5 × 10^6/mL) in 1× binding buffer, then 100 μL cell suspension was mixed with 5 μL Annexin V/fluorescein isothiocyanate in dark with room temperature for 5 min. Lastly, we

added 10 μL propidium iodide (PI) and 400 μL PBS to stain cells. We analyzed data using the FlowJo package.

Immunofluorescence and immunohistochemistry assays

We fixed skin tissue samples in 10% formalin solution, embedded them in paraffin, and sectioned them at 5 μm. Our team stained tissue sections with HE for histological detection. Our team used CD31 immunofluorescence staining to detect histopathological changes associated with angiogenesis. We performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) to identify apoptotic cells. Our team visualized sections using fluorescence (Nikon, Tokyo, Japan) under an Axiophot light microscope (Zeiss, Oberkochen, Germany), and photographed them by a digital camera.

Western blot assay

Skin tissues were lysed, and lysates were centrifuged at 12,000 rpm at 4 °C following addition of a protease inhibitor. The protein concentration was determined with a Pierce bicinchoninic acid assay (BCA) kit (Thermo Fisher). Proteins was separated by 10% SDS-PAGE and transferred to PVDF membranes. The primary antibodies used to assay protein expression were SIRT1 (1:600), FOXO1 (1:600) (all Santa Cruz Biotechnology, Dallas, TX, USA), Anti-GAPDH (1:1000, Sigma-Aldrich). Horseradish peroxidase-conjugated secondary antibody (1:1000, Abcam, USA). An ECL chemiluminescent kit (Millipore, Burlington, MA, USA) was used to read the bands.

Luciferase reporter assay

We created and cloned Wild-type (WT) and 3′-UTR mutant (MUT) *SIRT1*, as well as WT and MUT *circ-Astn1* into pMIR firefly luciferase-expressing vectors. We co-transfected the vectors into HEK293T cells once they reached 70% confluence, using 500 ng pMIR-SIRT1-wt/pMIR-SIRT1-Mut or pMIR-*circ-Astn1*-wt/pMIR-*circ-Astn1*-Mut combining with 50 nM miR-138-5p mimics with Lipofectamine 2000 Transfection Kit for luciferase assay. We assayed luciferase activity through Dual-Luciferase Reporter System (Promega, Madison, WI, USA). We performed 5 independent assays.

Tube formation assay

We performed EPC tube formation assay *via* Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Matrigel solution was mixed with ECMatrix diluent buffer then spread on μ-Slide plate and incubated it at 37°C for 1 h for matrix solution to solidify. Next, we added various treatment group EPCs (2×10^4 cells/well) to wells containing solid matrix and cultured them with EGM-2 medium at 37°C for a period of 12 h. Our team detected tube formation under inverted light microscope (×100) and evaluated 3 independent representative fields from each well to determine mean tube number.

Cell counting kit (CCK)-8 assay

EPC proliferation was evaluated using CCK-8 kit (BD Biosciences). Our team cultivated transfected cells in 96-well plates with Exos in HG conditions for 1 d in wells to which 10 μL CCK-8 reagent and 90 μL fresh culture medium was previously added. Absorbance was detected at 450 nm using a microplate reader following incubation at 37°C for two hours.

Statistical analyses

We denoted continuous parameters by means ± SD and employed one-way variance analysis (ANOVA) to compare data through GraphPad Prism (GraphPad, La Jolla, CA, USA). *P* value ≤ 0.05 indicated statistically significant.

RESULTS

ADSC and Exo characterization.

Isolated ADSCs have classical cobblestone-like morphology (Figure 1A). Immunofluorescence staining illustrated that ADSCs from samples of adipose tissue from mice were positive for expression of the mesenchymal cell surface markers CD29 (Figure B), CD44 (Figure C), CD90 (Figure D) and CD105 (Figure E), but negative for expression of the endothelial cell marker CD31 (Figure F) as well as von Willebrand Factor (Figure G). The results of Oil Red O staining (Figure H) together with alkaline phosphatase staining (Figure I) verified that isolated ADSCs owed both osteoblastic and adipocytic differentiation capacity. We concluded that ADSCs had the potential for multidirectional differentiation [20].

Exos were isolated by ultra-high speed centrifugation. Transmission electron microscopy revealed that ADSC Exos had spherical or cup-shaped morphology with diameter ranging in 50~120 nm (Figure 1J) as reported previously [21]. Western blotting suggested that ADSC Exos were positive for the Exo markers CD81 and CD63, which were cellular components (Figure 1K).

Exos derived from circ-Astn1-modified ADSCs play important roles in EPC function restoration *via* decreasing apoptosis under HG conditions.

To uncover the role of circRNAs in ADSC Exo-mediated restoration of EPC function under HG conditions, circRNA expression in ADSCs and fibroblast Exos was explored by RNA-Seq. Data verified that the contents of mmu_circ_0000101, mmu_circ_0008040, mmu_circ_0008061, and mmu_circ_0008099 were all significantly upregulated in ADSC Exos when compared with fibroblast Exos (Figure 2A). RT-qPCR analysis validated that mmu_circ_0000101, mmu_circ_0008040, mmu_circ_0008061 and mmu_circ_0008099 expressions in EPCs decreased after exposure to HG conditions (Figure 2B), with expression of mmu_circ_0000101 in particular decreasing most significantly. Consequently, mmu_circ_0000101 was selected for subsequent study. Mmu_circ_0000101 originated from *Astn1* gene exon 5, so mmu_circ_0000101 was also known as circ-Astn1. The entire mature spliced sequence length was 967 bp. The gene is on chromosome 1: 160432178-160441253 (Figure 2C).

Flow cytometry investigations showed that HG (30 mmol/L glucose) treatment promoted EPC apoptosis. Treatment with Exos from wild-type ADSCs suppressed HG-induced EPC apoptosis, and treatment with Exos from ADSCs overexpressing circ-Astn1 had more significant effect in suppressing HG-induced apoptosis of EPCs than Exos from wild-type ADSCs (Figure 2D and E), suggesting that circ-Astn1 functioned importantly in ADSC-Exo-mediated EPC protection under HG conditions. CCK8 detection verified that treatment with Exos containing high levels of circ-Astn1 had greater effect in restoring proliferative ability of EPCs under HG conditions (Figure 2F). We utilized tubule formation by EPCs in Matrigel-coated culture wells like *in vitro* angiogenesis model, and evaluated their potential by counting branch numbers that

constructed. HG conditions suppressed angiogenesis, and treatment with Exos containing high levels of circ-Astn1 were more effective in promoting angiogenesis of EPCs under HG conditions (Figure 2G-J).

The circ-Astn1-mediated miR-138-5p/SIRT1/FOXO1 signaling pathway protects EPCs under HG conditions by promoting angiogenesis.

Bioinformatics data showed that circ-Astn1 can regulate SIRT1 expression *via* inhibition of miR-138-5p. SIRT1 functions critically in promotion of angiogenesis by activating the FOXO1 signaling pathway [22]. To validate the interaction among circ-Astn1, SIRT1 and miR-138-5p, we created a luciferase reporter (LR) vector. The candidate miR-138-5p binding sites on circ-Astn1 as well as sites with point mutations inserted to prevent binding are shown in Figure 3A. Luciferase activity assay using 293T cells, which we transfected with MUT or WT circ-Astn1, verified that miR-138-5p suppressed circ-Astn1 activity (Figure 3B). RT-qPCR analysis suggested that circ-Astn1 overexpression suppressed miR-138-5p expression in EPCs (Figure 3C). Meanwhile tubule formation assay showed that upregulation of circ-Astn1 restored angiogenic differentiation ability under HG conditions, but miR-138-5p overexpression destroyed protective effect regarding circ-Astn1 (Figure 3D-G).

We next created the LR vector. Candidate miR-138-5p binding sites on SIRT1 3'-UTR and those with point mutations inserted to prevent binding were constructed (Figure 3H). We transfected 293T cell luciferase activities with MUT or WT SIRT1 3'-UTR, which verified that WT miR-138-5p suppressed SIRT1 activity (Figure 3I). RT-qPCR analysis illustrated that miR-138-5p overexpression suppressed FOXO1 and SIRT1 expression in both mRNA and protein level relating to EPCs (Figure 3J and K). However overexpression of SIRT1 promoted SIRT1 and downregulated FOXO1 expression even after miR-138-5p overexpression. Analysis of tubule formation verified that miR-138-5p upregulation decreased angiogenic differentiation ability, but overexpression of SIRT1 restored the angiogenic differentiation ability of EPCs (Figure 3L-O).

Exos from circ-Astn1-modified ADSCs hold high therapeutic effect, enhancing wound healing.

We investigated the influence of ADSC Exos on wound healing in full-thickness cutaneous wounds in mouse feet in a model of STZ-induced diabetes. Mice were treated by subcutaneous Exos injection from wild- or circ-Astn1-modified ADSCs, or an equivalent volume of PBS Exo diluent. Exos with high circ-Astn1 concentration accelerated wound closure significantly compared to PBS-treated control mice. The wounds treated with high circ-Astn1-containing Exos were almost closed by 14 d, while large regions of scarring were visible in both controls and circ-Astn1-knockdown-Exo (Figure 4A). Immunofluorescence with CD31 staining verified that microvascular development was more extensive with Exo treatments, specifically with high-circ-Astn1-containing Exos compared with the control group. But circ-Astn1-knockdown suppressed the therapeutic effect of Exo (Figure 4B and C). TUNEL staining suggested that circ-Astn1 Exos suppressed significantly skin tissue apoptosis comparing with control treatment. But circ-Astn1-knockdown suppressed the therapeutic effect of Exo (Figure 4D and E). HE staining also show that circ-Astn1 Exos treatment significantly promotion skin tissue wound healing comparing with control treatment. But circ-Astn1-knockdown suppressed the therapeutic effect of Exo (Figure 4F). RT-qPCR analysis validated that circ-Astn1 Exos significantly suppressed miR-138-5p expression (Figure 4G) but promoted SIRT1 (Figure 4H) and decreased FOXO1 (Figure 4I) expression in both mRNA and protein level when compared with controls.

DISCUSSION

Vascular deficits are fundamental factors regarding diabetes-related traits. Although former investigations revealed that proangiogenic wound healing phase was blunted by diabetes, detailed knowledge of functions regulating skin revascularization as well as capillary stabilization in diabetic wounds was missing [23]. Previous investigations revealed that Exos derived from ADSCs promote diabetic wound healing by regulating the disease microenvironment [4, 20]. There is also evidence that

circRNAs belong to a new RNA family that has been found to be broadly expressed, and have indispensable biological activities in regulating skin wound healing [18]. In this study we found a series of circRNAs, which RNA-Seq detection showed were abnormally expressed in ADSC Exos compared with fibroblast Exos. Among the abnormally-expressed circRNAs, we found that expression of mmu_circ_0000101 (circ-Astn1), mmu_circ_0008040, mmu_circ_0008061 and mmu_circ_0008099 were all increased significantly in ADSC Exos. Further study showed that circ-Astn1 decreased more significantly in EPCs after exposure to HG conditions. This suggested that ADSC Exos protected EPCs from HG-induced damage related to circ-Astn1 delivery.

Our *in vitro* experiments revealed that HG conditions induced promotion of EPC apoptosis and destroyed the ability of EPCs to differentiate into blood vessels. Transplantation of ADSC Exos exerted a protective effect in reversing HG-induced EPC damage. Increasing the circ-Astn1 content of Exos increased the protective effect. Bioinformatics analyses identified miR-138-5p as the circ-Astn1 downstream target, and this was confirmed by luciferase reporter experiments. A former study advised that overexpression of miR-138 aggravates HG-induced vascular cell damage [24]. Current investigation also found that circ-Astn1 overexpression decreased miR-138-5p expression. Meanwhile miR-138-5p overexpression reduced vascular EPCs differentiation, suggesting that circ-Astn1 protected against HG-induced EPC damage by miR-138-5p adsorption.

Additional bioinformatics results illustrated that SIRT1 was also a miR-138-5p downstream target and this was verified by luciferase reporter experiments. Sirtuin 1 (Sirt1) is a highly conserved nicotinamide adenosine dinucleotide (NAD)-dependent deacetylase, which plays regulatory role in metabolism and ageing [25]. miR-138-5p overexpression reduced SIRT1 expression. Overexpression of SIRT1 restored vascular differentiation of EPCs after miR-138-5p upregulation. Previous studies have suggested that SIRT1/FOXO1 pathway activity improves the stress microenvironment [26-28]. SIRT1 correlates to and deacetylates the forkhead transcription factor Foxo1. Moreover, previous studies have confirmed that SIRT1, a deacetylase that suppresses FoxO1

acetylation which is a crucial negative blood vessel development regulator, restraining anti-angiogenic activity [22, 29, 30]. Recently, it was reported that oxidative stress induces FoxO1 nuclear translocation which play an important in cell apoptosis regulation [26]. *In vivo* experiments validated that Exos originating from circ-Astn1-modified ADSCs functioned indispensably in restoring EPC function and promoting wound healing by promotion of angiogenesis and suppression of apoptosis. RT-qPCR analysis demonstrated that treatment with Exos containing high levels of circ-Astn1 reduced miR-138-5p expression and promoted SIRT1. This increase in SIRT1 Level suppressed FOXO1 expression, suggesting that Exos derived from circ-Astn1-modified ADSCs enhanced wound healing in a diabetic mouse model *via* miR-138-5p/SIRT1/FOXO1 axis regulation.

CONCLUSION

In conclusion, the present research indicated that Exos derived from circ-Astn1-modified ADSCs enhanced wound healing in a diabetic mouse model *via* miR-138-5p/SIRT1/FOXO1 axis induction. Our study verified the therapeutic effects of circ-Astn1-exosomes in STZ-induced diabetic wound healing model. However, more in-depth studies are required to determine the actual role of miR-138-5p/SIRT1/FOXO1 in wound healing.

ARTICLE HIGHLIGHTS

Research background

Wound healing impairment is a dysfunction consequence induced by hyperglycemia and its effect on endothelial precursor cells (EPCs) in type 2 diabetes mellitus (T2DM). There is increasing evidence showing exosomes derived from adipose-derived mesenchymal stem cells (ADSCs) exhibit potential to improve endothelial cell function along with the wound-healing process.

Research motivation

But the potential therapeutic mechanism of ADSCs exosome to wound healing in diabetic mice remains unclear.

Research objectives

To verified the effect of treatment with Exos from circ-Astn1-overexpressing ADSCs on high glucose (HG)-induced EPC dysfunction

Research methods

In this study, exosomes from ADSCs and fibroblasts were used for high-throughput RNA-Seq. ADSC-exosome-mediated healing of full-thickness skin wounds in diabetes mouse model was investigated. We utilized EPCs to investigate the therapeutic function of exosomes in cell damage and dysfunction caused by high glucose (HG). We utilized a luciferase reporter (LR) assay to detect interactions among circ-Astn1, sirtuin (SIRT)1 and microRNA (miRNA) (miR)-138-5p. We employed diabetic mice to verify circ-Astn1 therapeutic effect on exosome-mediated wound healing.

Research results

High-throughput RNA-Seq detection showed that circ-Astn1 expression was increased in ADSC exosomes compared with exosomes from fibroblasts. Exosomes contaning high circ-Astn1 concentration had enhanced therapeutic effect in restoring EPC function under HG conditions by promoting SIRT1 expression. circ-Astn1 expression enhanced SIRT1 expression through miR-138-5p adsorption, which was validated by LR assay along with bioinformatics analyses. Exosomes containing high concentrations of circ-Astn1 had better therapeutic effect on wound healing *in vivo* comparing to wild-type (WT) ADSC exosomes. Immunofluorescence and immunohistochemistry investigations suggested that circ-Astn1 enhanced angiopoiesis through exosome treatment of wounded skin as well as suppressing apoptosis through promotion of SIRT1 and decreased FOXO1 expression.

Research conclusions

In summary, we concluded that circ-Astn1 promoted ADSC-exosomes therapeutic effect and thus improved wound healing in diabetes *via* miR-138-5p absorption and SIRT1 upregulation. Based on our data, we advocate targeting the circ-Astn1/miR-138-5p/SIRT1 axis as potential therapeutic alternative regarding diabetic ulcers.

Research perspectives

more in-depth studies are required to determine the actual role of miR-138-5p/SIRT1/FOXO1 in wound healing.

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