

Exosomes from adipose-derived mesenchymal stem cells can promote fibroblasts proliferation, migration, and collagen synthesis via activating Wnt/ β -catenin signaling pathway during wound healing

Yu An

The Affiliated Hospital of Qingdao University

Cong Li

The Affiliated Hospital of Qingdao University

Quanchen Xu

The Affiliated Hospital of Qingdao University

Yu Sun

The Affiliated Hospital of Qingdao University

Zhiguo Wang (✉ qfywzg@126.com)

The Affiliated Hospital of Qingdao University <https://orcid.org/0000-0003-4401-4079>

Research

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Abstract

Background

Differentiation, migration, proliferation of skin fibroblasts are identified as the key factors during the cutaneous wound healing. Adipose-derived mesenchymal stem cells (ADMSCs) have been recorded as possible candidates for wound treatment because of their positive effect on the regeneration of many tissues. Exosomes derived from ADMSCs (ADMSC-Exos), an important signal transduction substance secreted by ADMSCs, have a similar role to ADMSCs in wound healing. However, the effects of ADMSC-Exos on cutaneous wound healing remain to be unclear. In this study, we tried to explore the role and mechanism of ADMSC-Exos during cutaneous wound healing.

Methods

Human skin fibroblasts (HSF) and ADMSCs were isolated from skin and adipose tissues of healthy person. ADMSC-Exos were purified from human ADMSCs culture medium by differential ultracentrifugation and identified by Electron microscopy, Nanoparticle tracking, and Western blotting assay. Fibroblasts were treated with different concentrations of ADMSC-Exos. The proliferation and migration abilities of fibroblasts were analyzed by CCK-8 assay and scratch method. The synthesis of collagen type I (Col-I), collagen type III (Col-III), and α -smooth muscle actin (α -SMA) in fibroblasts was assessed by real-time quantitative polymerase chain reaction and Western blotting assay. A tensional wound model on rat back was used to evaluate the effect of ADMSC-Exos on wound healing. The expression levels of Wnt2b and β -catenin were analyzed by Western blotting and immunohistochemical assay.

Results

ADMSC-Exos were successfully obtained. ADMSC-Exos could significantly promote the migration and proliferation ability of fibroblasts in a dose-dependent manner in vitro. Compared with the treatment without ADMSC-Exos, the expression levels of Col-I and Col-III in fibroblasts treated with ADMSC-Exos were significantly increased, while the expression level of α -SMA is decreased. Besides, the enhanced expression of Wnt2b and β -catenin proteins confirmed the activation of the Wnt/ β -catenin signaling pathway.

Conclusions

ADMSC-Exos can promote fibroblasts proliferation, migration, and collagen synthesis in a dose-dependent manner and may play a positive role in skin wound healing through Wnt/ β -catenin signaling pathway. So our study elucidates part of the mechanism of ADMSC-Exos in wound healing, which

illustrates the therapeutic potential of ADMSC-Exos as a new therapeutic approach to promote skin wound healing.

Background

Cutaneous wound healing is a complex and continuous process that includes hemostatic, inflammatory, proliferative, and remodeling stages. Various reasons, such as high wound tension, infections, radiation damage, and metabolic diseases, lead to prolonged wound healing time, causing physical or mental pain and severe treatment burden to patients [1, 2]. Fibroblasts are the most abundant cells in the dermis, which have the ability to synthesize and remodel extracellular matrix (ECM). During different wound healing stages, the integration of proliferation, differentiation, and migration of skin fibroblasts plays an important role [3–5]. Therefore, the regulation of fibroblasts is a very significant target in scar treatment.

Recently, cell therapy has received much attention for its applications in skin tissue repair [6]. ADMSCs have become one of the most promising stem cell populations identified so far because they not only have the effect of enhancing tissue regeneration can as the other mesenchymal stem cells (MSCs) but can be harvested in larger quantities easily with minimal damage to the donor site [7–9]. Numerous studies have reported that ADMSCs can promote the proliferation and migration of fibroblasts and epidermal cells during tissue injury, and promote the formation of new blood vessels in ischemic tissue injury, and finally accelerate tissue regeneration and wound healing [10–12]. However, due to the high clearance rate of stem cells in vivo and the potential carcinogenic risk, the clinical application of stem cells is limited [13]. Recent studies have shown that one of the mechanisms of action of MSCs is through the paracrine pathway, among which the discovery of exosomes is more important [14, 15].

Exosomes enclosed by lipid membranes are a type of extracellular vesicle secreted by cells, which contain a variety of microRNA, proteins, cytokines, lipids, and unedited RNA [16], so they have similar information-transmitting functions as the source cell and can transport a variety of cellular components to target cells. Many cells have been proved to secrete exosomes, such as lymphocytes, tumor cells, MSCs, oligodendrocytes, epithelial cells, etc. [16]. Exosomes are formed in cells through the mechanism of endocytosis [17]. Exosomes play an important role in regulating different physiological and pathological processes, such as substance transmission, cell survival, cell apoptosis, and cell proliferation. Besides, exosomes can regulate angiogenesis, immune regulation, and reduce ischemia-reperfusion injury [18]. Meanwhile, exosomes have the following characteristics: long-time activity, easy to transport, low immunogenicity, easy to control the concentration and the contents change with the microenvironment [19]. Researchers believe that exosomes are the paracrine effectors of MSCs. Some studies have reported that exosomes derived from MSCs participate in a wide range of biological processes by affecting tissue responses to injury, infection, and disease [16]. However, the mechanism is poorly understood [17].

Wnt/ β -catenin signaling pathway is a classical Wnt signaling pathway, which plays an important role in wound healing [20]. Our previous studies show that Wnt-responsive stem cells residing in the oral mucosa

and bone are responsible for the oral wound healing[21, 22]. There is also evidence that mesenchymal stem cell-derived exosomes may play a role in promoting cardiovascular disease healing by activating the Wnt/ β -catenin signaling pathway [23]. However, the relationship between ADMSC-Exos and the Wnt/ β -catenin signaling pathway in wound healing remains unclear.

In this study, we extracted ADMSC-Exos by hypervelocity centrifugal method and investigated its effect on normal skin fibroblasts to explore its role in wound healing process. We also used a SD rat model of tension skin defect to investigate the potential relationship between ADMSC-Exos and Wnt/ β -catenin signaling pathway in wound healing. Therefore, this study aims to clarify the regenerative effects of ADMSC-Exos in cutaneous wound repair.

Materials And Methods

1. Isolation and characterization of ADMSCs.

This study was approved by the Ethics Committee of Qingdao University and informed consent was obtained from the participants. Human adipose tissue, which is obtained from liposuction surgery in the affiliated hospital of Qingdao University, was digested with 0.075% collagenase type I (Invitrogen, USA) for 45 minutes after rinsing blood components. The digestion was stopped using DMEM (Hyclone, China) complete medium, then the solution centrifuged at 1500 r/min (Beijing Times Beili, China) for 10 minutes. ADMSCs deposited at the bottom of the centrifuge tube. After resuspended with medium containing 15% FBS (Procell, China), the ADMSCs solution was transferred to culture dishes. A small number of ADMSCs were observed 1 week later, and a large number of ADMSCs were observed half of a month later. Images of typical areas were viewed through a microscope (Olympus Corporation, Japan).

The third-generation ADMSCs were rinsed by PBS for 3 times and digested with 0.25% trypsin. After the ADMSCs were prepared into $1 \times 10^6 \text{ L}^{-1}$ cell suspension, they were divided into 6 parts and moved to the centrifuge tube, respectively. Then PE-anti-human CD29, 488-anti-human CD44, PE-anti-human CD45, PE-anti-human CD90, 488-anti-human CD105 and PBS were added into centrifuge tubes, 5 μl each, and incubated at room temperature and dark for 30 min. After centrifugation at 1000 r/min for 5 min, the samples were washed with PBS for 3 times, and resuspend with 200 μL PBS for Flow cytometry. The data was analyzed with Flowjo Software 7.6.1.

Induction of osteoblasts and adipoblasts of ADMSCs. After the third generation of ADMSCs were fused and grew to 80–90%, the induction medium of osteoblasts or adipoblasts was replaced. Alkaline phosphatase staining was performed on the 9th day of induction and Alizarin red S staining was performed on the 21st day of induction to observe osteogenesis. Oil red O staining was performed on the 14th day of induction to observe the lipid formation. The results were observed and recorded under microscope (Olympus Corporation, Japan).

2. Isolation of fibroblasts

Skin fibroblasts were isolated from the foreskin tissue after male circumcision which was obtained from the male department of the Affiliated Hospital of Qingdao University. The isolated full-thickness skin tissue was rinsed 3 times with sterile water for injection to remove visible blood components. The outer epidermis and subcutaneous tissue were removed by sterilizing surgical scissors, and only the dermis was retained. The remaining white tissue was cut into square tissue blocks of about 0.5 x 0.5 cm² in size and evenly spread on the bottom of the petri dish (1cm interval). An appropriate amount of complete medium with 15% FBS (Procell, China) was added to ensure that the medium submerged the tissue blocks and the bottom of the tissue blocks was close to the bottom of the petri dish. The petri dishes were transferred to the constant temperature incubator (SANYO, Japan) for culture at 37°C, 5% CO₂. The cell culture medium was changed every 2 days. In general, a large number of primary cells could be seen to gather around the tissue block in about 14 days, and the primary passage could be achieved.

3. Isolation and characterization of ADMSC-Exos

Exosome extraction was based on our previous study and modified appropriately [24]. In short, when the cells were fused to 70%~80%, they were replaced with serum-free medium for 36 h. After collecting the ADMSCs media, the media were centrifuged for 10 min at 300 g, 10 min at 2000 g, and 30 min at 10 000 g, respectively, to remove dead cells and large cell debris. The supernatant was filtered through a 0.22µm-pore Needle filter (Corning,China) to remove cell debris and large vesicles. The volume of supernatant was reduced from approximately 250–500 mL to 30 mL. The supernatant was then ultracentrifuged at 100 000 g for 70 min at 4°C using 70Ti rotor (Beckman Coulter, USA). The resulting pellets were resuspended in 40ml PBS and ultracentrifuged at 100 000 g for 70 min at 4°C using 100Ti rotor (Beckman Coulter, USA). The obtained exosomes were resuspended with 200 µl PBS, stored at -80°C and used in the experiments as soon as possible. Exosome suspension concentration was determined according to the instructions of the BCA protein concentration assay kit (Procell, China).

Purified exosomes were fixed with 1% glutaraldehyde in PBS (pH 7.4). After rinsing, a 20 µl drop of the suspension was loaded onto a formvar/carbon-coated grid and negatively stained with 3% (w/v) aqueous phosphotungstic acid for 1 min. The results were observed by transmission electron microscopy. After 20 µl of purified vesicles were diluted to 200 µl with PBS, the particle diameter distribution was measured with nanoparticle tracking analysis (NKT, China).

Exosome-specific protein markers CD9 and CD63 were measured by Western-blot assay. After protein sampling, electrophoresis and membrane transfer, successful PVDF (Millipore, USA) bands were obtained. The PVDF protein was placed face up in the antibody incubator box, and 3% skim milk powder (Yili, China) was added for sealing for 1.5 h. After sealing, the membrane was washed with TBST for 3 times, 10min/ time. Incubation of primary antibody: preprepared primary antibody solution (CD9 1:1500; CD63 1:1500) were immersed in PVDF membrane and placed in a refrigerator at 4°C overnight. After primary antibody incubation, the membrane was washed with TBST for 3 times, 10min/ time, and the residual solution of primary antibody was washed off. Incubation secondary antibody: a goat anti-rabbit secondary antibody solution (1:1500) (Elabscience, China) prepared with 3% skim milk powder was

immersed in PVDF membrane and incubated for 1.5 h. After secondary antibody incubation, the membrane was washed with TBST for 3 times, 10min/ time, and the residual secondary antibody was washed. Finally, the PVDF membrane was developed and photographed, and the image analysis software ImageJ was used for analysis.

4. Experimental study on wound healing in animal models

To evaluate the effects of ADMSC-Exos on wound healing, a total of 9 male SD rats (270g ~ 290g) (Pengyue Laboratory Animal Breeding Co. Ltd, China) were randomly divided into blank control group, PBS treatment group and ADMSC-Exos treatment group. The rats were anesthetized by intraperitoneal injection with 10% Chloral hydrate (0.3 ml/100g). Full-thickness round wounds of equal sizes ($0.8 \times 0.8 \text{ cm}^2$) were aseptically generated in the middle of the back and a ring-shaped rubber ring was sewn around the wound for 10 days to give the wound a proper tension. After 10 days, the ring-shaped rubber ring was removed. The rats were housed separately after surgery. On the 1st, 5th and 10th day after injury, 200 μL PBS containing 100 μg ADMSC-Exos was injected subcutaneously and intradermally with a 1ml disposable syringe. The PBS group was injected with the same amount of PBS buffer solution, while the control group was not treated. On days 0, 3, 7 and 14 after injury, the wound size was taken by camera (Canon, China) and measured by Image-Pro Plus 6.0 software. The rats were sacrificed 14 days after operation. Skin tissue was cut 2 mm around the healed wound and paraffin-embedded immunohistochemistry was performed.

5. Immunohistochemical analysis

After fixation with formaldehyde, the excised skin samples from the wound sites was dehydrated in a low to high concentration alcohol solution and immersed in xylene to obtain a transparent tissue mass. After paraffin embedding, the tissue blocks were cut into sections of 4 μm . After paraffin sections were dewaxed with xylene and anhydrous ethanol, antigen repair was performed with citric acid antigenic repair buffer (PH 6.0). The endogenous peroxidase was blocked with H_2O_2 and the tissue was sealed with 3% BSA. After removing the blocking solution, the prepared primary antibody Wnt2b (1: 200), β -catenin (1: 500), Col-I (1: 100), Col-III (1: 100), α -SMA (1: 200) were added to the tissue sections, and the sections were incubated overnight at 4°C . The tissue sections were washed with PBS (PH 7.4) for 3 times, 5min each. After the sections were slightly dried, the secondary antibody (HRP label) of the corresponding species to the primary antibody was added to cover the tissues, and the tissues were incubated at room temperature for 50min. The tissue sections were washed with PBS (PH 7.4) for 3 times, 5 minutes each time, and freshly prepared DAB color solution was added for staining. The color developing time was controlled by observation under microscope. After restaining the nucleus with hematoxylin, the tissue sections were dehydrated and sealed. Microscopically, the images were collected using an FSX100 microscope (Olympus, Japan) for further analysis.

6. Migration assay

The effects of ADMSC-Exos on fibroblasts migration were evaluated by scratch assay. Briefly, fibroblasts were seeded in 6-well plates at 5×10^5 cells/well and cultured normally. When the cell confluence reached

90%, the medium was replaced with a fresh FBS-free medium after two washes with PBS, then the confluent cell monolayer was scratched using a sterile 200 µl pipette tip. Different concentrations of ADMSC-Exos (0 µg/ml, 25 µg/ml, 50 µg/ml, and 100 µg/ml) were added to the wells. Images were recorded at 0 h, 12 h, and 24 h after the monolayer scratches. The migration area was measured by using Image J software (Medical Cybernetics, USA) and assessed as follows: migration area (%) = $(A^0 - A^n)/A^0 \times 100\%$, where A^0 represents the initial wound area (t = 0 h) and A^n represents the residual area of the wound at the time of measurement (t = n h).

7. Cell growth assay

Fibroblasts were seeded into 96-well plates at a density of 2×10^3 cells/well for 6 h. The cells were then divided into 4 groups and treated with ADMSC-Exos at the gradient concentration of 0 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml, respectively. Each group had 6 repeat Wells. The well plates were removed at 12 h, 24 h and 48 h, and 10 µl CCK-8 reagent was added to the plates. The plates were incubated in an incubator at 37°C for 2 h without light. The absorbance value at 450 nm was detected with a microplate analyzer, and the proliferation of cells in each well was compared.

8. Real-Time PCR analysis

The expression of each gene was detected by qRT-PCR. The fibroblasts in the logarithmic growth phase were inoculated into 6-well culture plates with 5×10^5 cells / well. After the cell density grew to about 70%, the medium was sucked out and washed twice with PBS. Then the cells were divided into 4 groups, which were treated with different concentrations of exosomes (0 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml), respectively, and cultured for 36 h for subsequent experiments.

RNA extraction from fibroblasts was performed using Trizol® reagent (Takara, China) with 1 ml/well following the manufacturer's instructions. Then, 300 ng of RNA was reverse transcribed into cDNA using the Prime Script RT Reagent kit (Takara, China). Quantitative PCR was performed using an RT-PCR system (Takara, China), with SYBR Premix Ex Taq II (Takara, China) in a 10 µl volume of the PCR reaction solution. After initial denaturation at 95°C for 90 s, the amplification conditions were as follows: denaturation at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 30 s, with a total of 40 cycles. GAPDH was used as the reference gene for the calculations. The results were expressed relative to GAPDH with the comparative CT method. Oligonucleotides were synthesized by Integrated DNA Technologies (Takara, China). The primer sequences are listed in Table 1.

Table 1
Primers used for qRT-PCR

Gene	Sequences
Col-I (Col1a1 homo)	Forward: TAGGGTCTAGACATGTTTCAGCTTTG Reverse: CGTTCTGTACGCAGGTGATTG
Col-III (Col3a1 homo)	Forward: TCAGGCCAGTGGAATGTAAAGA Reverse: CACAGCCTTGCGTGTTTCGATA
α -SMA (Acta2 actin alpha 2 homo)	Forward: CTCTGGACGCACAACTGGCATC Reverse: GGCATGGGGCAAGGCATAGC
Human GAPDH (Shenggon, China)	

9. Western blot assay

Western blot assay was conducted following the standard protocols. The fibroblasts were seeded in a 6-well plate according to the density of 5×10^6 cells/ well. After the cells grew to a density of about 70%, the medium was extracted and the cells were treated with different concentrations of exosomes (0 μ g/ml, 25 μ g/ml, 50 μ g/ml and 100 μ g/ml), respectively, which were incubated in an incubator at 37°C and 5% CO₂ for 36 h to detect the collagen synthesis of fibroblasts. In addition, fibroblasts were spread in a 6-well plate according to the density of 5×10^6 cells/well. After the cells grew to a density of about 70%, the culture medium was extracted and replaced with 2 ml culture medium containing 50 μ g/ml exosomes, PBS buffer and serum-free DMEM medium, respectively. After incubation in an incubator at 37°C and 5% CO₂ for 36 h, the cells were used to detect the protein expression of Wnt2b/ β -catenin. After the cells were treated with lysate (protein lysate RIPA: protease inhibitor=100: 1), the concentration of the protein sample was determined with the BCA kit (Procell, China) according to the instructions. After adjusting the protein concentration, Loading Buffer of 1/4 sample volume was added and mixed by eddy oscillation. The protein was denatured by metal bath at 95°C for 5min. Wnt2b (1: 3000) (Abcam, UK), β -catenin (1: 5000) (Abcam, UK), Col-I (1: 2500) (Abcam, UK), Col-III (1: 2500) (Elabscience, China), α -SMA (1: 2000) (Abcam, UK), and GAPDH (1: 2000) (Elabscience, China) were used as primary antibodies. The corresponding secondary antibody also uses goat anti-rabbit antibodies (Elabscience, China). The remaining steps were the same as the Western blot experiment in the identification of exosomes. Signals were monitored by the Enhanced Chemiluminescence Detection System (Millipore, Bedford, MA).

10. Statistical analysis

All the experimental data were collected and analyzed by GraphPad 7 and SPSS17.0 software. T-test was used for comparison between single groups, and one-way analysis of variance was used for comparison between multiple groups (> 2). The experimental data were expressed as mean \pm standard deviation ($\bar{X} \pm S$). The difference was considered statistically significant when $P < 0.05$.

Results

1. Isolation and characterization of ADMSCs

After initial isolation and culture for 36 h, ADMSCs were observed via a microscope and most of the adherent cells were in spindle-like shape during cell culture. After the 3rd generation, the morphology of ADMSCs gradually stabilized to long spindle-shaped, similar to fibroblasts, with larger nuclei (Fig. 1A). Flow cytometry of ADMSCs showed positive expression of stem cell specific markers CD29, CD44, CD90, and CD105, while negative expression of hematopoietic marker CD45 (Fig. 1E ~ I). Osteogenic induction of ADMSCs showed that the alkaline phosphatase staining was blue-purple at the 9th day (Fig. 1B). On the 21st day of induction, the cells showed lamellar growth with unclear structure, and red calcium nodules were observed in the extracellular matrix by alizarin red S staining (Fig. 1C). The adipogenic induction of ADMSCs showed that small vacuoles with good light transmittability began to appear in the cytoplasm of adipogenic cells on the 4th day of adipogenic induction, and a large number of vacuoles of different sizes with good light transmittability appeared on the 14th day of adipogenic induction. The oil red O staining was orange red (Fig. 1D). The above results proved that ADMSCs were successfully extracted

2. Isolation and characterization of ADMSC-Exos

The cup-shaped morphology of ADMSC-Exos was observed by transmission electron microscopy (TEM) ($\times 100\,000$ magnification) (Fig. 2B). Exosomes purified from ADMSCs' culture supernatants were characterized by a Particle size analyzer and the results showed the exosomes are circular membrane-bound vesicles with a diameter of 40 to 100 nm (Fig. 2C), which was consistent with the previously reported exosome size distribution [25]. Western blots also showed that the exosomal markers CD9 and CD63 were expressed in ADMSC-Exos (Fig. 2D). These results indicated that exosomes from human ADMSCs were successfully isolated.

3. ADMSC-Exos accelerate cutaneous wound healing in rats

We locally injected 100 μg of ADMSC-Exos into the rat model of full-thickness skin defect at different time points (1 d, 5 d, 10 d), and photographed the wound healing at different time points (0 d, 3 d, 7 d, 14 d). The wound healing rate was statistically analyzed using Image-Pro Plus 6.0, and the results showed that the wound healing rate in the ADMSC-Exos injection group was significantly faster than that in the PBS injection group and the blank control group, indicating its positive effect on the wound (Fig. 2E, F).

4. ADMSC-Exos can promote skin fibroblasts' proliferation and migration

In order to study the effect of ADMSC-Exos on the proliferation and migration of fibroblasts, we treated fibroblasts with different concentrations of exosomes (0 $\mu\text{g}/\text{ml}$, 25 $\mu\text{g}/\text{ml}$, 50 $\mu\text{g}/\text{ml}$, 100 $\mu\text{g}/\text{ml}$), and the results of CCK-8 method showed that, with the increase of exosome concentration, the proliferation ability of fibroblasts was significantly increased compared with the blank control group (Fig. 3C), and the

difference of proliferation ability among different concentrations was statistically significant ($P < 0.05$). Meanwhile, the scratch test results also showed that the migration rate of fibroblasts increased with the increase of exosome concentration (Fig. 3A, B), and the difference of migration rates among different concentrations was statistically significant ($P < 0.05$). ADMSC-Exos showed concentration dependence on the proliferation and migration of fibroblasts, in which 100 $\mu\text{g}/\text{mL}$ ADMSC-Exos had the most significant effect. These results suggest that ADMSC-Exo can promote the proliferation and migration of fibroblasts.

5. ADMSC-Exos promoted collagen synthesis and decreased α -SMA synthesis in fibroblasts

The mRNA and protein expression levels of Col-I, Col-III and α -SMA in fibroblasts under the effect of ADMSC-Exos were analyzed by Western blot and qRT-PCR. The results showed that ADMSC-Exos could promote collagen synthesis by stimulating the synthesis of Col-I and Col-III in fibroblasts, and this promoting effect was enhanced with the increase of ADMSC-Exos concentration. At the same time, the expression of α -SMA was decreased, indicating that the myofibroblast differentiation was inhibited by ADMSC-Exos (Fig. 4A, B, D). Interestingly, when the concentration of ADMSC-Exos reached the high concentration of 100 $\mu\text{g}/\text{mL}$, the collagen synthesis ability of fibroblasts decreased compared with the low concentration, but it was still higher than 0 $\mu\text{g}/\text{mL}$. Immunohistochemical results of rat wounds also showed that the expressions of Col-I and Col-III were increased and α -SMA was decreased in the ADMSC-Exos group (Fig. 4C).

6. ADMSC-Exos may promote wound healing by activating Wnt/ β -catenin signaling

To investigate the potential molecular mechanism of ADMSC-Exos in wound healing, we performed two experimental protocols in vivo and in vitro. Firstly, ADMSC-Exos was applied to normal skin fibroblasts, and the protein expressions of wnt2b and β -catenin were determined by Western-blot assay. Secondly, we performed an immunohistochemical assay to detect the protein expression of Wnt2b and β -catenin in the scar tissue of rats under the action of ADMSC-Exos. We found that the expression of Wnt2b and β -catenin both in vivo and vitro experiments was noticeably enhanced in the ADMSC-Exos group compared with the control group (Fig. 5A ~ C), suggesting that ADMSC-Exos may promote wound healing by activating Wnt/ β -catenin signaling pathway.

Discussion

Exosomes play an important role in intercellular communication [26]. In recent years, the basic research and translational application of exosomes in tumorigenesis and tissue repair have become a research hotspot [27–30]. Exosome-based therapy is emerging as a promising alternative to cell-based therapy [31]. Although there are many studies on the role of ADMSC-Exos in wound healing, little is known about its

mechanism due to the complex composition of exosomes [32]. In this study, we investigated the effect of ADMSC-Exos on human normal fibroblasts and the therapeutic effect of rat wound model, and explored its potential mechanism of action. Assessing the effect of ADMSC-Exos on fibroblasts, which play a key role in wound healing, we found that ADMSC-Exos can promote the proliferation and migration of fibroblasts; upregulate the expression of Col-I and Col-III, and downregulate the expression of α -SMA. In evaluating the effect of ADMSC-Exos on wound healing, we found that ADMSC-Exos could accelerate wound healing, regeneration, and reduce scar formation; lead to the activation of Wnt/ β -catenin signaling pathway.

Normal wound healing is one of the most complex biological processes, requiring the coordinated cooperation of multiple cells and the accurate coordination of various biological and molecular events [33]. Skin grafts, laser therapy and the topical application of some growth factors or gene therapy are often used to accelerate wound healing and reduce scar formation. However, these methods may lead to atrophic scar, abnormal pigmentation, skin necrosis and other adverse consequences, and can not completely eliminate scar [34]. Therefore, we still need to study new methods to promote wound healing. Stem cell therapy for tissue repair has been extensively studied over the past few decades. However, stem cell therapies have had limited success in clinical use due to a lack of adequate evidence of safety and efficacy [35]. As mentioned above, exosomes contain the components of the cells from which they are derived and have the function of transmitting similar information. Simultaneously, exosomes also have the advantages of low immunogenicity, long time activity and easy transportation, so they are considered a promising way to use for wound healing without the risk of immune rejection and tumor.

Exosome extraction by ultracentrifugation (UC) is widely used for exosome extraction due to its high purity and low price [36]. In this study, we applied the UC to extract ADMSC-Exos. The nanoparticle tracking system showed that the diameters of our extracted ADMSC-Exos ranged from 40 to 100 nm (Fig. 2C). We also observed the typical cup shape of exosomes under TEM (Fig. 2B) and Western blot analysis confirmed that exosomes showed high expression of CD9 and CD63 (Fig. 2D), which is consistent with previous study [34, 37]. In summary, we successfully identified exosomes extracted from ADMSCs by UC. Meanwhile, Karlien et al. found that local injection of ADMSCs could save drugs and achieve a high wound closure rate, which was superior to caudal vein injection [38]. Therefore, we used local injection method in our study.

Fibroblasts are the main effector cells in soft tissue wound healing. Their proliferation and migration are necessary for wound contraction, collagen synthesis, and tissue remodeling [39]. In this study, we evaluated the effect of ADMSC-Exos on the behavior of fibroblasts in vitro. The results indicate that these nanoparticles can significantly enhance their proliferation migration and collagen synthesis, suggesting that fibroblast activation is a mechanism by which ADMSC-exos stimulates wound healing. However, when the concentration of exosomes reached 100 μ g/ml, the ability of ADMSC-Exos to enhance collagen synthesis of fibroblasts was weakened. The specific mechanism remains to be further studied, but it can be shown that the function of exosomes is related to concentration. The expression of α -SMA has been considered to be a marker of myofibroblast differentiation [40], as static myofibroblasts do not strongly

express α -SMA. So, over-differentiation of fibroblasts is an important aspect of α -SMA continued expression. For the decreased expression of α -SMA in our research, we predicted that the application of ADMSC-Exos to the wound increased the migration rate of fibroblasts from the peripheral to the center of the wound surface, thus reducing the surface tension. At the same time, we speculate that ADMSC-Exos may be able to inhibit the conversion of fibroblasts to myofibroblasts, thereby inhibiting the excessive formation of scars. These results suggest that ADMSC-Exos has a positive role in promoting wound healing and reducing scars.

Wnt/ β -catenin signaling pathway plays an important role in the regulation of cell proliferation, apoptosis and differentiation, and participates in the multipotency of stem cells [20]. Increasing evidence has confirmed that the activation of the Wnt/ β -catenin signaling pathway plays an essential role in the proliferative phase of wound healing [41, 42]. β -catenin, a subunit of the cadherin protein complex, exerts as an integral component of the classic Wnt signaling pathway [42]. Exosomes are an emerging platform and a critical factor in facilitating Wnt secretion and transport. Zhang et al. found that exosomes from human umbilicalcord mesenchymal stem cells can deliver the Wnt4 to the target cells and activate the β -catenin nuclear translocation, which has a positive effect on wound healing [43]. Ma et al. found that ADMSC-Exos could increase the expression of Wnt2B and β -catenin proteins in HaCaT, which is treated with hydrogen peroxide to simulate skin damage[37]. In this study, the elevated expression of β -catenin in vitro under the action of ADMSC-Exos suggested that Wnt/ β -catenin signaling may be related to the underlying mechanism of ADMSC-Exos in wound healing. Moreover, we test the expression on rat tissue after the action of ADMSC-Exos, which showed the same results. Taken together, these results suggested the positive role of ADMSC-Exos through Wnt/ β -catenin signaling in cutaneous wound healing. However, more studies should be carried out in the aspect of the association between Wnt/ β -catenin signaling pathway and cutaneous wound healing. Our previous studies have shown that Wnt-responsive oral mucosal stem cells are activated in response to oral mucosal injury, thereby accelerating wound healing [21]. In this study, our results showed that ADMSC-Exos can promote skin wound healing through Wnt signaling pathway, so whether ADMSC-Exos can also promote skin stem cells through Wnt signaling pathway in the process of wound healing remains to be further studied. Our previous studies have also shown that Wnt-responsive stem cells can regulate periodontal membrane fibrosis and alveolar bone density to adapt to the change of mechanical stress [22]. Therefore, it remains to be further studied whether the effect of ADMSC-Exos in skin wound healing can also regulate the effect of mechanical tension by regulating Wnt-responsive stem cells.

Conclusion

In this study, ADMSC-Exos was used as an intervention factor to conduct in vivo and in vitro experiments, and it was found that ADMSC-Exos may play a positive role in wound healing through the Wnt/ β -catenin signaling pathway. ADMSC-Exos has the properties of activating fibroblasts, such as promoting proliferation, migration and collagen synthesis, and inhibiting the excessive differentiation of fibroblasts to myofibroblasts to promote the healing of tension wound tissue. Our results indicate that ADMSC-Exos

may represent a new therapeutic tool for wound healing and provide a theoretical basis for clinical application.

Abbreviations

ADMSCs: adipose-derived mesenchymal stem cells; ADMSC-Exos: exosomes derived from ADMSCs; Col-I: collagen type I; Col-III: collagen type III; ECM: extracellular matrix; HSF: human skin fibroblasts; α -SMA: α -smooth muscle actin; MSCs: mesenchymal stem cells; UC: ultracentrifugation

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University and conducted in accordance with its guidelines. Patients who provided adipose tissue and skin were informed and given their consent.

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Yu An conducted the main experiment, analyzed the data, and wrote the first draft. Cong Li was responsible for manuscript rewriting, manuscript proofreading, data sorting, picture processing, and manuscript submission preparation. Therefore, Yu An and Cong Li made the same contribution in this paper. Quanchen Xu and Yu Sun participated in the experiment and achieved good results. Zhiguo Wang supervised the whole experimental research and the writing of the paper. All authors read and approved the final manuscript.

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Figures

Figure 1

ADMSCs were detected by light microscopy, induced differentiation and cytometry. (A) Results of the third generation of ADMSCs observed under light microscope (x40 magnification); (B) The alkaline phosphatase staining of ADMSCs on day 9 of osteogenesis is blue-purple, as shown by arrow (x100 magnification). (C) On day 21 of osteogenic induction, the cells were lamellar with unclear structure, and red calcium nodules were observed in the extracellular matrix stained with alizarin red S, as shown by arrow (x100 magnification); (D) On day 14 of adipogenesis, fat vacuoles were stained orange by oil red O, as shown by the arrow (x200 magnification); (E~I) Flow cytometry showed positive expression of CD29, CD44, CD90 and CD105, and negative expression of CD45.

Figure 2

Isolation of fibroblasts; Exosomes were successfully isolated from ADMSCS medium; ADMSC-Exos promotes wound healing. (A) On day 7, sporadic primary fibroblasts were seen crawling out from around the self-tissue mass (x40 magnification). (B) Exosomes were observed by transmission electron microscopy (TEM) (x100 000 magnification). (C) Nanoparticle tracking analysis (NTA) showed that the ADSC-Exos distribution diameter ranged from 40 nm to 100nm. (D) The expression of the exosome protein markers CD9 and CD63 was confirmed by Westernblot assay. (E, F) As can be seen from the wound images at different time points, the wound shrinkage rate of the AMDMSC-Exos group was significantly faster than that of the PBS group and the blank control group. At the 14th day, the remaining wound area was significantly reduced compared with the other two groups.

Figure 3

ADSC-Exos promote fibroblasts cell proliferation and migration. (A, B) Representative images from the scratch wound assay and quantitative analysis of cell migration in each group at 12 h and 24 h. (C) CCK-8 analysis shows the proliferation results of fibroblasts at different concentrations of ADMSC-Exos. The comparison between the two groups was performed using the student T-test. * $P < 0.05$. Scale bars, 200 μ m.

Figure 4

In vitro and in vivo, ADMSC-Exos affects the synthesis of collagen and α -SMA in fibroblasts. (A) The qPCR analysis of the mRNA expression of Col-I, Col-III, α -SMA in human fibroblasts treated with different concentrations of ADMSC-Exos. (B, D) Western blot analysis of the protein expression level of Col-I, Col-III, α -SMA in fibroblasts treated with different concentrations of ADMSC-Exos. (C) Representative images of immunohistochemistry showed Col-I, Col-III, α -SMA expression in the designated treatment group. The

comparison between the two groups was performed using the student T-test. * $P < 0.05$, compared with 0 $\mu\text{g/ml}$ group. Scale bar = 500 μm .

Figure 5

ADMSC-Exos may promote wound healing via activating the Wnt/ β -catenin signaling pathway. (A, B) Western blot analysis of the protein expression level of Wnt2b and β -catenin in fibroblasts treated with 50 $\mu\text{g/ml}$ ADMSC-Exos, PBS and non-FBS DMEM for 36 hours. (C) Representative images of immunohistochemistry showed Wnt2b and β -catenin expression in the designated treatment group. The comparison between the two groups was performed using the student T-test. * $P < 0.05$, compared with the PBS and DEME group. Scale bar = 500 μm .