Adipose Mesenchymal Stem Cell-derived Exosomes can Promote Cell Proliferation, Migration and Collagen Synthesis of Human Skin Fibroblasts

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Abstract

Objective To explore the effects of human adipose mesenchymal stem cell derived exosomes (ADMSC-exos) on cell migration, proliferation and collagen synthesis of human skin fibroblasts. Methods Normal human skin fibroblasts（NSFB）and ADMSCs were isolated from normal patient skin and adipose tissues. ADMSC-exos were extracted by ultracentrifugation. The size of the exosomes was analyzed with a particle size analyzer, the exosomes were observed with an electron microscope, and the exosome specific surface marker proteins CD9 and CD63 were detected by Western blot assay. Human skin fibroblasts were cultured and treated with different concentrations of ADMSC-exos (0, 25, 50 and 100μg/ml) in media. The proliferation and migration abilities of fibroblasts were analyzed by CCK-8 assays and scratch assays, respectively. Type I、III collagen were detected by Real-time fluorescence quantitative PCR (qRT-PCR) and Western blot assay. Results ADMSC-exos were successfully obtained. ADMSC -exos could significantly promote the cell migration and proliferation ability of fibroblasts in a dose-dependent manner in vitro. The mRNA and protein expression level of Type I, III collagen in HSFB treated with ADMSC-exos increased significantly compared with that without ADMSC-exos. Conclusion our findings suggest that ADMSC-exos can promote human skin fibroblasts proliferation, migration and collagen synthesis. Our research may provide new insights into the therapeutic goals of skin wound healing.

Keywords

Adipose mesenchymal stem cells; Exosomes; Fibroblasts.

1. Introduction

Skin wound healing is a complex and continuous process that includes hemostatic, inflammatory, proliferative, and remodeling stages. Various reasons, such as infection, radiation damage, and basic metabolic diseases, lead to prolonged wound healing time, often leading to physical and mental pain and severe clinical diagnosis and treatment burden on patients[1, 2]. During different wound healing stages, the integration of proliferation, differentiation, and migration of wound cells (such as skin fibroblasts, skin epidermal cells, etc.) plays an important role in skin tissue repair. The main feature of wound healing is re-epithelialization and proliferation and migration of fibroblasts and keratinocytes[3]. Although skin wound healing has made many advances, there are no effective therapies for specific mechanisms in skin healing.

Recently, cell therapy has received much attention for their applications in skin tissue repair[4]. Different types of cells including fibroblasts, keratinocytes, adipose-derived stromal vascular fraction cells, platelets, and stem cells have been used to improve wound repair in clinical trials. Mesenchymal stem cells (MSCs) are a kind of pluripotent stem cells with multi-directional differentiation ability[5]. They play a vital role in enhancing the ability of tissue regeneration. They are one of the most widely used stem cells in the field of regenerative medicine because of their essential role in augmenting the regenerative capacity of many tissues. Previous studies have found that MSCs play an important role in neovascularization (including wound healing) of ischemic tissue[6]. Mesenchymal stem cells can

be divided into bone mesenchymal stem cells, umbilical cord blood mesenchymal stem cells (UCB-MSCs), and adipose mesenchymal stem cells (ADSCs) according to different tissue sources. ADSCs have attracted the attention of many researchers due to their diverse sources, ease of access, and low damage to the donor site. Numerous studies have determined that MSCs promote the proliferation and migration of fibroblasts and epidermal cells during tissue injury, and can promote the formation of new blood vessels in ischemic tissue injury, that is, promote wound healing[7, 8]. At the same time, the mechanism by which MSCs play a role is through a paracrine pathway, and the discovery of exosomes has important significance for this. Exosomes are a vesicle structure of lipid bilayers released by almost all human cell types with a diameter of 30-100mm and are involved in cell–cell communication and intracellular signaling[9, 10]. They contain various bioactive cargos including protein, lipid, messenger ribonucleic acid (mRNA), microRNA, and deoxyribonucleic acid (DNA) fragments[11]. Many studies have found that MSC-derived exosomes have significant effects on tissue regeneration and wound healing in various animal models[12].

In this study, we isolated exosomes from adipose stem cells and added them to skin fibroblasts to reveal the role of ADSC-exos in wound healing.

2. Materials and Methods

2.1 Cell Culture

Human adipose tissue was digested with 0.075% collagenase type I for 45 minutes after liposuction surgery in the affiliated hospital of Qingdao university. The digestion was stopped using DMEM (Hyclone, China) complete medium and centrifuged at 1500rpm/min for 10 minutes to obtain cell debris particles. Fat mesenchymal stem cells deposited at the bottom of the centrifuge tube were transplanted to the culture, and a small number of fat MSCs were observed 1 week later, and a large number of fat MSCs were observed half of month later. Images of typical areas are viewed through a microscope (Olympus corporation, Tokyo, Japan).

As mentioned above, skin fibroblasts (HSFB) were isolated from the foreskin tissue after male circumcision which was obtained from the male department of the Affiliated Hospital of Qingdao University, and sorted and cultured in DMEM (Hyclone, China) containing 10% HSFB (Procell, China) at 37℃, 5%CO_2.

2.2 Isolation and Characterization of Exosomes

Exosome extraction was performed as described above[13]. In short, when the cells were fused to 70%~80%, they were replaced with serum-free medium for 36 h. ADMSC culture medium was collected and centrifuged at 300 g for 5 min, 2000g 10min and additionally centrifuged at 10000 g for 30 min to remove the elevated cells. The supernatant was subjected to filtration on a 0.22um-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 ℃ using 70Ti rotor (Beckman Coulter). The resulting pellets were resuspended in 40ml PBS and ultracentrifuged at 100,000 g for 70 min at 4 ℃ using 100Ti rotor (Beckman Coulter). Exosomes obtained were preserved at -80 ℃ by resuscitating the precipitate with 200ul PBS. 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 ul drop of the suspension was loaded onto a formvar/carbon-coated grid, negatively stained with 3 % (w/v) aqueous phosphotungstic acid for 1 min, and observed by transmission electron microscopy.

After 20ul of purified vesicles were diluted to 200ul with PBS, the particle diameter distribution was measured with a particle size analyzer.

Exosome specific protein markers were measured by Western-blot assay.

2.3 Migration assay

The effects of ADMSC-Exos on HSFB migration were evaluated in a scratch assay. Briefly, HSFB were seeded in 12-well plates at 1 × 105 cells/well and cultured normally. When the cell confluence reached 90%, the medium was replaced with fresh FBS-free medium after two washes with PBS, the confluent cell monolayer was scratched using a sterile 200-μl pipette tip, the cells were washed, and the edge of the scratch was smoothed with PBS. Different concentrations of ADMSC-Exos (0, 25, 50 and 100μg/ml) were added to the wells. Images were recorded 0, 12 and 24 h after the monolayers were scratched. 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).

2.4 Cell growth assay

HSFB were seeded at 2,000 cells/well in 96-well plates and cocultured with different concentrations of ADMSC-Exos (0, 25, 50 and 100μg/ml). Cell growth was analyzed 6h, 12 h and 24 h after exosome treatment. The optical density (OD) was measured at 450 nm using a microplate reader. The data shown are representative of three independent experiments.

2.5 Real-Time PCR analysis

The expression of each gene was detected by qRT-PCR. The HSFB in the logarithmic growth phase were inoculated into 6-well culture plates with 5×10^5 cells / well. After the complete medium was cultured for 24 hours, they were replaced with conditioned medium and serum-free medium with corresponding concentrations of exosomes. Continue Incubate for 36h.

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 a RT-PCR system (Takara, China), with SYBR Premix Ex Taq II (Takara, China) in a 20-μl volume of the PCR reaction solution. The amplification conditions after an initial denaturation step for 90 s at 95 °C were 40 cycles of 10 s at 95 °C for denaturation, 10 s at 55 °C for annealing and 30 s at 72 °C for elongation. 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	
Col -l（Col1a1 homo）	Forward：TAGGGTCTAGACATGTTCAGCTTTG Reverse：CGTTCTGTACGCAGGTGATTG
Col - Ⅲ（Col3a1 homo）	Forward：TCAGGCCAGTGGAAATGTAAAGA Reverse：CACAGCCTTGCGTGTTCGATA
Human GAPDH（Shenggon, China）	

2.6 Western blot assay

Western blot assay was conducted in accordance with the standard protocols. Protein concentration was quantified with the BCA Protein Assay Kit (Procell, China). The primary antibodies were as follows: CD9 (ABclonal, China) at a 1:1500 dilution, CD63 (ABclonal, China) at a 1:1500 dilution, Wnt2b (Abcam, UK) at a 1:3000 dilution, Col -l (Abcam, UK) at a 1:2500 dilution, Col -Ill (Elabscience, China) at a 1:2500 dilution, GAPDH (Elabscience, China) at a 1:2000 dilution. While the corresponding secondary antibodies were CD9, CD63, Wnt2b, Col –l, Col – Ill, (goat anti-rabbit, AS014, 1:3000; Elabscience). Signals were monitored by the enhanced chemiluminescence detection system (Millipore, Bedford, MA).

2.7 Statistical analysis

All experiments were independently performed in triplicate. GraphPad 7 and SPSS17.0 software was applied for statistical analysis. All data were presented as $\bar{X}\pm S$ deviation and the differences among multiple groups (>2) were analyzed by one-way analysis of variance. The differences were considered to be statistically significant as a result of $P < 0.05$.

3. Results

3.1 Isolation and characterization of ADMSC-exos

After primarily isolated and cultured for 36 h, ADMSC cells were observed via a microscope (×40 magnification) and most of the adherent cells were in spindle-like shape during cell culture (Fig. 1A). The cup-shaped morphology of the exosomes was observed by transmission electron microscopy (TEM) (100,000×) (Fig.1B). Exosomes purified from ADMSCs culture supernatants were characterized by Particle size analyzer and the results showed the exosomes are circular membrane-bound vesicles with a diameter of 40 to 150 nm (Fig. 1C), which was consistent with the previously reported exosome size distribution. Western blots also showed that the exosomal markers CD9 and CD63 (Fig. 1D) were expressed in exosomes. These results indicated that exosomes from human ADMSCs were successfully isolated and were consistent with previously defined exosomes. The protein concentration was determined to be 1.0 μg/ul using a BCA protein assay kit (Procell, china).

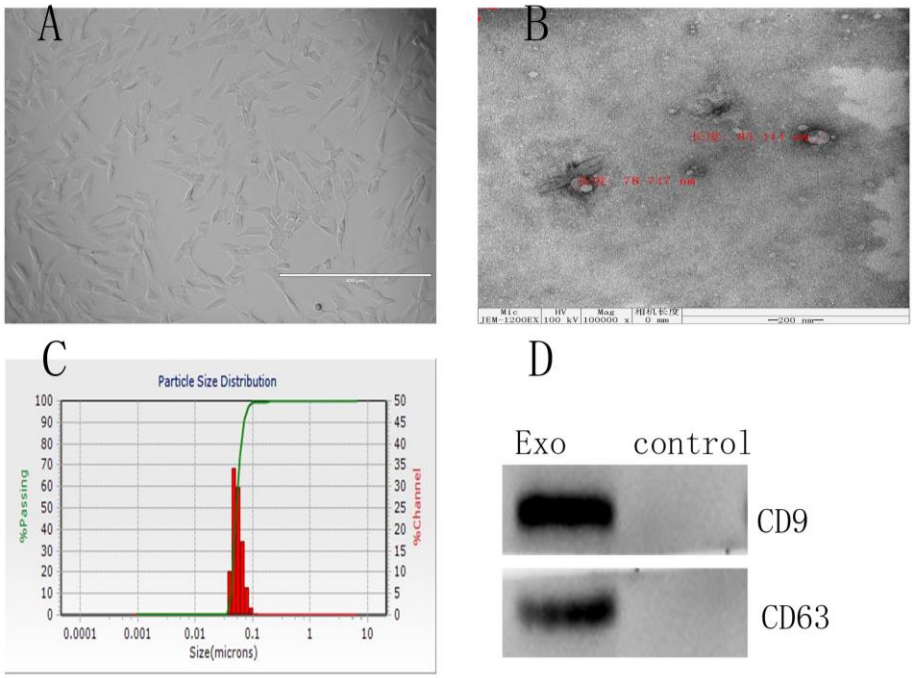

Fig. 1 Successful isolation of exosomes from ADMSC culture medium. (A) ADMSCs exhibited the typical fibroblastic morphology, as shown in the picture. (B) The ultrastructure of ADMSC-Exos under transmission electron microscopy. (C) The size distribution profile of ADMSC-Exos by Particle size analyzer. (D) The expression of the exosome markers CD9 and CD63 was confirmed by Western blot assy.

3.2 ADMSC-exos can promote skin fibroblast proliferation, migration

To assess the effects of ADMSC-exos on fibroblasts, the HSFB were treated with different concentrations of ADMSC-exos in vitro functional assays. A scratch wound assay was applied to measure the effect of ADMSC-exos on the migration of HSFB. The result showed that exosomes treatment markedly enhanced the motility of HSFB, as determined by the migration area (Figure 2A). The proliferation of HSFs was quantified by CCK-8 analysis. The result revealed that exosomes stimulation resulted in a significant increase in HSFB proliferation (Figure 2B). All these data suggest that ADMSC-exos can enhance fibroblast function, which is equal with previous study.

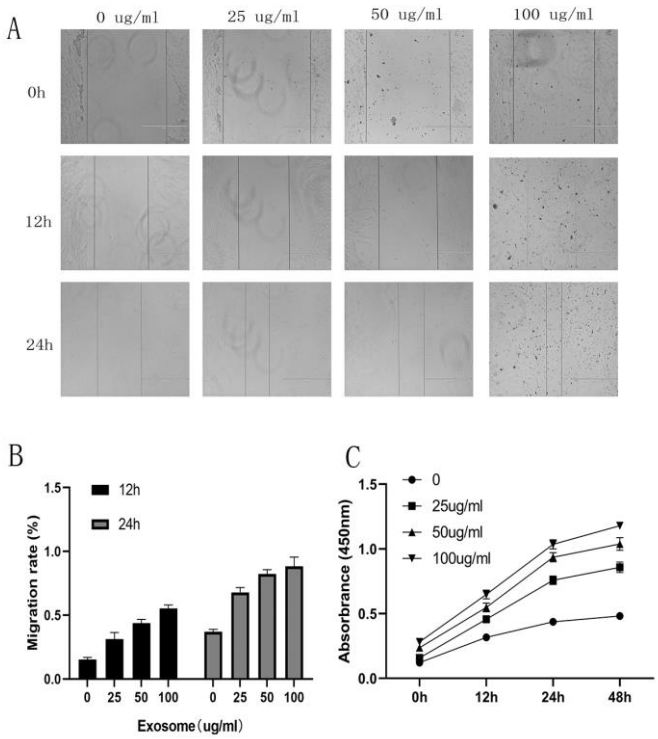

Fig. 2 ADMSC-Exos promote HSFB cell proliferation and migration. (A、B) Representative images from the scratch wound assay and quantitative analysis of cell migration in each group at 24 h. (P< 0.05 and P < 0.05). (C) CCK-8 assay.

3.3 ADMSC-exos can promotes collagen synthesis in vitro

Further in vitro studies were performed to determine whether exosomes can affect collagen synthesis in fibroblasts. After stimulating fibroblasts with ADMSC-derived exosomes, the gene and protein expression of Col-l, Col-III (Fig. 3) were significantly increased in a dose-dependent manner. Intrestingly, when the concentration of ADMSC-exos reached a high concentration of 100 ug / ml, the collagen synthesis ability of HSFB played an opposite role compared with the lower concentration group (Figure 3A-C).

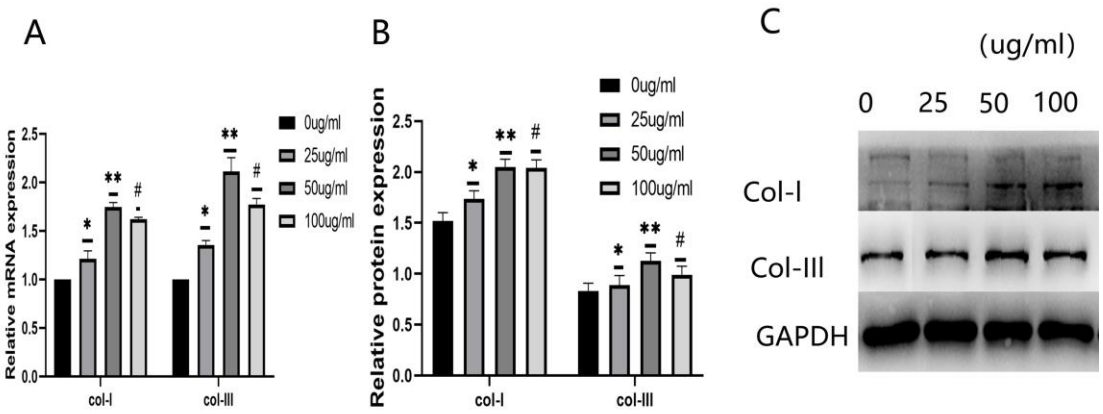

Fig. 3 ADMSC-exos activate intracellular collagen secretion in HSFB. (A) The gene expression of Col -l, Col-III. (B、C) The protein expression level of Col -l, Col-III. The data in these groups represented at least three independent experiments, expressed by mean ± SEM of three replicates. *P< 0.05、**P< 0.05 and $^{\#}$P < 0.05, compared with ahead group;

4. Discussion

Exosomes are released from cells and implicated in many biological and pathological process. A novel role of exosomes as a cell-cell communicator has been identified in many research fields due to its capacity of carrying messenger RNA, microRNA, and proteins[14], which could be transferred into target cells[15, 16]. According to previous reports, ultracentrifugation (UC), ExoQuick, or total exosome separation reagent (TEI) are the mainstream methods for exosomal extraction. Among them, ultracentrifugation (UC) is more known than other purification methods for its high purity of the isolated exosomes[17]. In this study, we applied the UC to extract ADMSC-derived exosomes. Then we were used TEM, NTA and Western blot assay to confirm the existence of exosomes and describe individual extracellular vesicles[18]. The results from TEM (Fig.1B) and NTA (Fig.1c) showed that we successfully isolated particles in exosomes (40-150nm)[19], which showed morphological characteristics consistent with exosomes. We also observed the typical cup-shaped morphology exosomes under TEM (Fig.1B). We also found that exosomes obtained by UC showed high expression of CD9 and CD63 as identified by western blot assay (Fig.1D), which is consistent with previous study[20, 21]. In summary, we identified the extracellular vesicles extracted by UC as exosomes.

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[22]. In this study, we also 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. Therefore, the beneficial effects of UCB-exos on wound healing may be mainly because of their promoting endogenous fibroblasts functions. If intervention measures are taken during early wound healing to accelerate wound healing and prevent excessive proliferation and accumulation of myofibroblasts, instead of taking remedial measures after scar formation, it may be one of the methods for treating scars.

The role of exosomes from stem cells in promoting tissue repair has been reported by many sources[12, 13, 23]. For example, it has been reported that exosomes derived from human amniotic epithelial cells can accelerate wound healing and inhibit scar formation via promoting the migration and proliferation of fibroblasts, which has the same results with ours. Similarly, Human adipose mensenchymal stem cells derived exosomes also can optimize the characteristics of fibroblasts in a dose-dependent manner and improve the genes expression of collagen I, III to accelerate cutaneous wound healing, which has similar results with ours.

In short, exosomes secreted by human adipose stem cells are easy to obtain and can be effectively used for research and clinical treatment. Exosomes can optimize the characteristics of fibroblasts, such as promoting fibroblast migration, proliferation, and collagen synthesis in dose-manner independent, thereby promoting wound healing in soft tissues.

5. Conclusion

In conclusion, we had extracted exosomes from human adipose stem cells successfully. At the same time, we verified the promoting effects of ADMSC-exos to human skin fibroblasts in vitro experiment. Our findings suggest that ADMSC-exos, a kind of stem cells product, may represent a new therapeutic tool in wound healing.

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