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Curcumin-Induced Adipose-Derived Stem Cell Exosomes Alter Adipogenic Differentiation Capabilities

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Abstract

Adipose-Derived Stem Cells (ADSCs) have multi-directional differentiation potential; however, their efficiency in differentiating into mature adipocytes remains low, which is a limitation for clinical and research applications of stem cell therapy. Curcumin has been reported to promote the proliferation of adipose-derived stem cells, but the effects of curcumin on the secretion of ADSC exosomes and the function of exosomes on adipogenic differentiation have not been assessed. Therefore, the objective of the present study was to investigate the exosome of ADSCs stimulated with curcumin and the consequence for adipogenic differentiation. Human ADSCs were isolated by the adherence method and characterized according to adipogenic, osteogenic, and chondrogenic differentiation ability. Exosomes were extracted from the supernatant of the third-generation ADSCs, and characterized by transmission electron microscopy and western blotting. Curcumin (10 μmol/L) was added to the complete culture medium 3 days before the extraction, and the influence of the exosomes on adipogenic differentiation was assessed. The human ADSCs showed mesenchymal stem cell characteristics and could differentiate into mature adipocytes, osteoblasts, and chondrocytes. Exosomes secreted by curcumin-treated ADSCs showed typical exosome characteristics and enhanced cell migration compared with that of exosomes in the untreated group. Moreover, curcumin-treated exosomes increased the number of fat cells produced after adipogenic differentiation but not the volume of fat cells. Overall, these results indicate a beneficial role of exosomes from ADSCs on adipogenesis and ADSC activity, providing new insight into the processes of fat accumulation and potential new strategies for improving the expansion of ADSCs for their clinical use in regenerative medicine.

Keywords: Exosome; Adipose-derived stem cell; Fat accumulation; Differentiation; Tissue regeneration; Curcumin

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Introduction

As a seed cell, stem cells have the ability to expand and differentiate into a variety of cell lineages, making them attractive tools for tissue regeneration and cell therapy. Transplantation of autologous stem cells prepared *in vitro* can promote tissue repair. A large number of *in vitro* and *in vivo* experimental studies have demonstrated that Adipose-Derived Stem Cells (ADSCs) are Mesenchymal Stem Cells (MSCs) with multi-differentiation potential, and are thus now widely used in tissue engineering and cell-free treatments [1]. Compared with other types of stem cells, ADSCs have advantages of convenient access, sufficient source, retrievable materials, little resistance to donors, little pain, no ethical issues, and potential for use in autologous transplantation [2]. Moreover, the requirements of the culture medium are low, and the storage conditions have been standardized. Accordingly, ADSCs have attracted substantial attention in recent years, becoming a hotspot in stem cell research for tissue engineering [1]. However, the efficiency of their differentiation to adipocytes remains low, which can hinder clinical applications.

The adipose tissue is a key system to regulate energy balance and lipid metabolism in the human body. Fat cells store excess glucose and fatty acids in the form of lipid droplets from the circulation when the body's energy intake is sufficient. This process can increase the volume of fat cells and contribute to expansion of the adipose tissue [3]. Therefore, in-depth study of the regulation mechanism of the proliferation and differentiation adipocytes has important clinical significance for not only improving stem cell therapy with ADSCs, but also for understanding the formation of

body fat and regulation of the body's immune function and growth.

Peroxisome Proliferator-Activated Receptor Gamma (PPARγ) is a nuclear hormone receptor that regulates cell proliferation and differentiation at the transcriptional level by affecting the function of fatty acids and their derivatives [4]. In this way, activated PPARγ not only promotes adipocyte differentiation and increases the number of adipocytes but also increases the expression of related genes in the adipose tissue [5], including genes involved in processes such as lipogenesis, transport, storage, and oxidation. PPARγ has been identified to take a central position in the transcriptional regulatory network of adipocyte differentiation and to play an integral role in the early stages of adipocyte differentiation [6]. Importantly, all transcriptional regulators must initiate the adipocyte differentiation process in the presence of PPARγ [7].

Several factors have been identified to influence the proliferation and adipogenic differentiation of ADSCs. Exosomes are extracellularly secreted nanovesicles of approximately 100 nm that play a key role in cell communication [8,9] and have also been identified as an important contributor to the beneficial paracrine effects of ADSCs therapy [9,10]. Various *in vitro* experiments have shown that ADSCs can be affected by many factors during adipogenic differentiation and can secrete exosomes in the microenvironment. In addition, curcumin is a phenolic pigment extracted from the roots of turmeric (Curcuma longa), which has strong anti-inflammatory and anti-oxidative properties and can stimulate the proliferation of embryonic neural progenitor cells [11]. Moreover, curcumin has been suggested to promote the osteoblast differentiation of rat MSCs by upregulation of HO-1 [12,13]. Recent studies have also shown that curcumin plays an important role in the proliferation of ADSCs. However, the regulation of exosomes in the conversion of ADSCs into adipocytes remains unknown and the influence of curcumin on promoting the secretion of ADSC exosomes is unclear.

Accordingly, the aim of the present study was to determine whether exosomes have a positive effect during ADSCs through an *in vitro*-based approach and to analyze the effects of curcumin-induced ADSC exosomes on lipid size and number, and related protein expression during adipogenic differentiation [14].

Materials and Methods

Cells and cell culture

Human adipose tissues were obtained from patients undergoing tumescent liposuctions after receiving informed consent. All experiments were approved by First Affiliated Hospital of Wenzhou Medical University, and all clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. Human ADSCs were isolated from the human adipose tissues and characterized as previously described. After isolation, ADSCs were maintained in a serum-free medium specifically formulated for the growth and expansion of human MSCs. Passage 3-6 ADSCs were used in the experiments. The culture medium was replaced every three days. Cell cultures were maintained in a 5% CO2 humidified incubator at 37K°C (Heracell 150i, Heraeus, Langenese, Germany).

Cell viability assay

The viability of ADSCs treated with various concentrations of curcumin *in vitro* was measured by the Cell Counting Kit-8 (CCK-8) assay according to the manufacturer instructions. ADSCs were seeded in 96-well plates at a density of 1×10^4 cells/mL and incubated overnight in a 5% CO2 humidified incubator at 37°C. The following day, the cells were incubated with curcumin at different concentrations for an additional 48 h and 72 h, and cell viability was determined. CCK-8 assays were performed in sextuplicate and repeated three times independently. Based on these results, 10 mmol/L was determined as the optimal working concentration of curcumin for subsequent experiments.

Isolation and characterization of ADSC-derived exosomes

Fetal bovine serum was centrifuged using an ultracentrifuge at 100,000 × g for 4 h to remove the exosomes, and 10% of exosome-free serum was added to Dulbecco's Modified Eagle Medium (DMEM) as the culture medium. Exosome purification from ADSCs was performed according to a differential centrifugation approach. The ADSCs were treated with curcumin (10 μmol/L) for 72 h or left untreated as the control. The medium was collected, and the exosomes were isolated through multistep centrifugation at 300 × g for 10 min, 2000 × g for 10 min, and 10,000 × g for 30 min at 4°C. The supernatants from the final centrifugation were ultracentrifuge at 110,000 × g for 3 h at 4°C. After removing the supernatants, the exosome pellets were washed in a large volume of ice-cold Phosphate-Buffered Saline (PBS) and centrifuged at 110,000 × g for an additional 3 h at 4°C. The final pellets were resuspended in PBS, and the amount of exosome proteins was assessed by a Bicinchoninic Acid (BCA) assay (Thermo Fisher Scientific, Inc.). Transmission electron microscopy (FEI Tecnai Spirit TEM T12) and western blotting (with antibodies against exosome markers Alix, CD63, and CD9) were used to identify the collected exosomes as reported previously [8,15].

Transwell migration assay

For the transwell migration assay, ADSCs (1×10^5 cells/well, three replicates per group) were seeded into the upper chambers of 24-well transwell plates (Corning, Corning, NY, USA) with 8-μm pore filters and cultured for 12 h [16]. The lower chamber was replaced with serum-free DMEM supplemented with or without ADSC-exosomes (100 μg/well) or curcumin-induced ADSC-exosomes (100 μg/well) [17]. After 12 h, the cells attached on the upper surface of the filter membranes were cleaned by a cotton swab, and the cells at the bottom of the membrane were fixed with 4% paraformaldehyde and stained by 0.5% crystal violet (Sigma Aldrich, St. Louis, MO, USA) to visualize the migrated cells under an optical microscope (Leica DMI6000B, Germany). Three fields from each well were randomly selected for quantitative determination of the color-positive area using ImageJ software (Media Cybernetics, Rockville, MD, USA).

Adipogenic induction

The third-generation ADSCs were inoculated into a 6-well plate at 2×10^5 cells/well, grown to 80% confluence, and the adipogenic induction medium (containing 1 μmol/L cortisol, 0.5 μmol/L isobutyl methyl xanthine, 10 ng/mL insulin, and 1 μmol/L dexamethasone) was added. Every 3 days, the plates were supplemented with or without ADSC-exosomes (50 μg/well) or curcumin-induced ADSC-exosomes (50 μg/well). After 14 days of culture, Oil-red-O staining was performed to determine the degree of adipogenesis based on the formation of lipid droplets. In brief, the culture medium in the culture dish was aspirated, the cells were gently rinsed in PBS twice, and 4% paraformaldehyde was added and let stand for 30 min at room temperature. The fixative solution was then aspirated, and the cells were washed in deionized water twice before adding the Oil-red-O dye solution. The cells were incubated for 30 min at room temperature, washed twice with deionized water, and observed under the microscope.

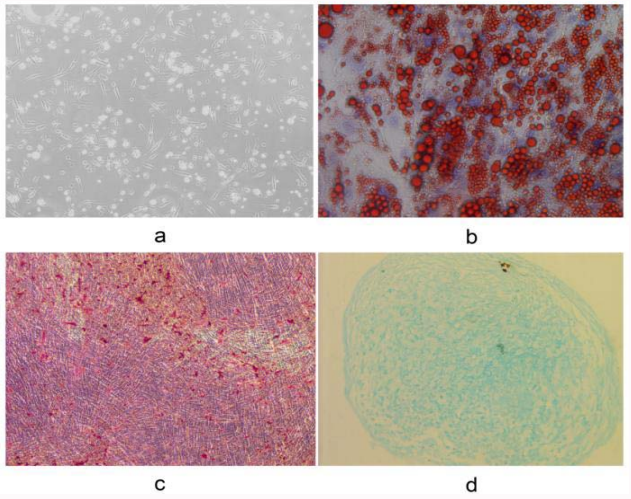

Figure 1: Morphology and multipotential differentiation of adipose-derived stem cells. (a) is a spindle-shaped growth of stem cells from the third generation; (b) is lipid-induced oil red O staining, lipid droplets are stained red; (c) is osteogenic-induced alizarin red staining, calcified nodules are red; (d) is cartilage Induction of Alcian blue staining, cells in the nodules and surrounding aggregates were blue.

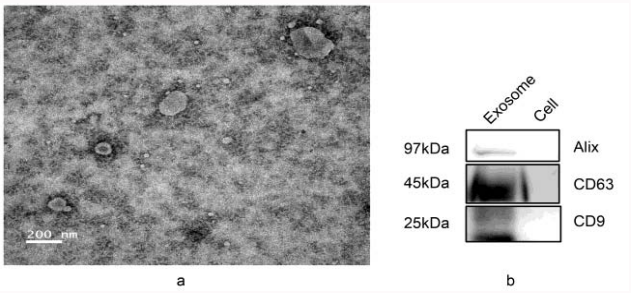

Figure 2: Characterization of ADSC-Exo. (a) Representative TEM image of ADSC-Exo; (b) Western blot analysis of the exosomal markers (Alix, CD63, and CD9).

Western blotting

An appropriate amount of lysate was added to the cells of each group, the cells were fully lysed and centrifuged, and the supernatant was obtained as the whole protein extract. After measuring the protein concentration of each sample with the BCA protein concentration measuring kit, a suitable volume of the loading buffer was added, and the protein was denatured by heating in a 100°C water bath for 10 min. Samples containing total protein (30 μg protein per lane) were subject to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 2 h at 100 V and then transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% (w/v) nonfat milk, and the blots were incubated overnight at 4°C with the Polyclonal primary antibody rabbit anti-human PPARγ (1:500). The GAPDH antibody (Santa Cruz, Dallas, TX, USA) was used as an internal control. The intensity of the protein bands was quantitatively analyzed with Image Lab software and normalized to the intensity of GAPDH. The western blot assays were repeated three times to ensure reproducibility.

Statistical analysis

All results are presented as mean ± standard error. Statistical comparisons were performed using one-way analysis of variance, followed by the Bonferroni test for multiple comparisons, and with the Dunnett T3 test for samples with unequal variances, using SPSS 19.0 (SPSS Inc., Chicago, IL, USA); P values <0.05 were considered statistically significant.

Results and Discussion

Morphological and phenotypic identification of ADSCs

Fat conversion is a highly coordinated sequence of biological events involving the coordination of many cell types and signaling molecules for the ultimate goal of maintaining energy metabolism in the body. Adipose cells differentiate from ADSCs in a lipogenic environment in the adipose tissue [18,19], and their metabolism is closely related to the microenvironment of the tissue [20]. ADSCs also account for a certain proportion of the adipose tissue, and their paracrine function plays a regulatory role throughout the tissue [17,21]. In this study, we investigated the effects of curcumin-induced ADSC exosomes on adipogenic differentiation.

Human ADSCs could be easily expanded *in vitro* and showed fibroblast-like morphologic features (Figure 1a). To verify their multipotent differentiation potential, ADSCs were incubated in medium known to induce differentiation to adipogenic osteogenic or cartilage formation lineages. Oil-Red-O staining confirmed adipogenic differentiation by the formation of intracellular lipid droplets (Figure 1b) and Alizarin red confirmed osteogenic differentiation by staining of matrix mineralization (Figure 1c). In addition, Alcian blue staining confirmed the capacity of cartilage differentiation (Figure 1d). The ADSCs obtained from all patient samples showed similar multipotent differentiation potential.

ADSCs are efficient exosome producers

Transmission electron microscopy was used to identify exosomes from the ADSC culture medium. Ultrastructural analysis of ADSC-derived exosomes confirmed that they have a diameter of around 100 nm (Figure 2). Expression of the exosome markers CD9, CD63, and Alix was then confirmed by western blotting (Figure 2).

Curcumin

Pretreated ADSC exosomes promote the migration and

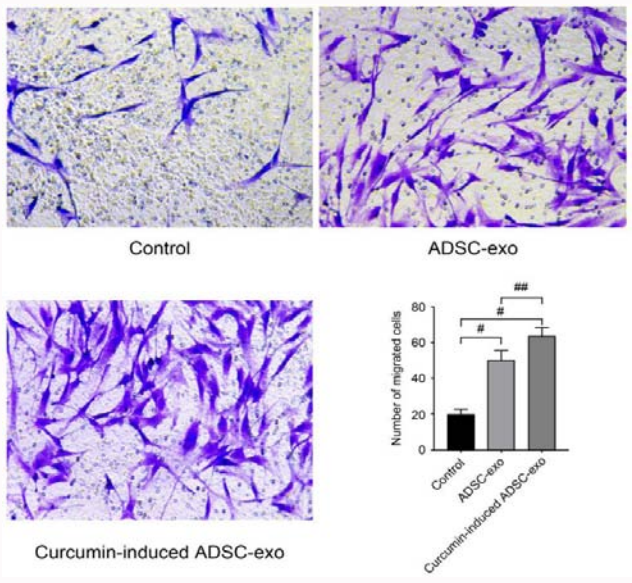

Figure 3: The number of cells that migrated into the denuded areas was counted and the results were quantitated by counting migrated cells in five randomly chosen fields for each replicate.

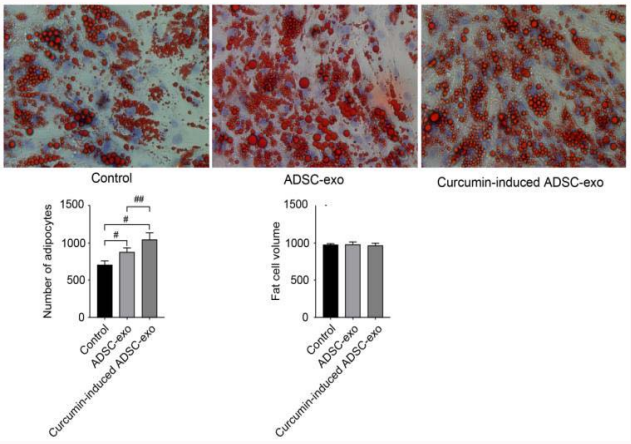

Figure 4: Randomly select five fields to calculate the average number and volume of fat cells. The number of adipocytes after adipogenic differentiation of the two experimental groups was significantly different from that of the control group, but the volume of adipocytes was similar in three groups.

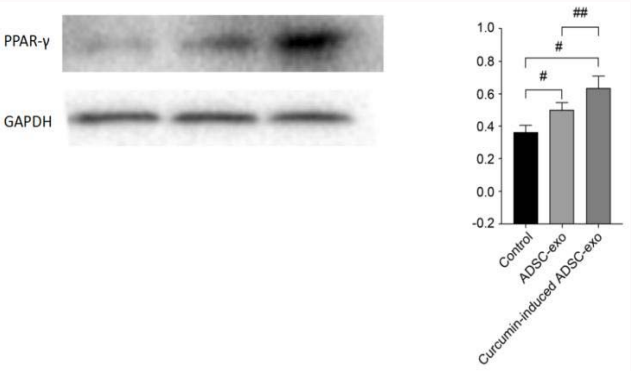

Figure 5: Difference in the protein content of PPAR-γ in fat cells after 14 days.

adipogenic differentiation of ADSCs.

Transwell experiments showed that curcumin-induced ADSC-exosomes significantly increased the ability of ADSCs to migrate, with no such effect observed for untreated ADSCs (Figure 3). This finding indicates that exosomes can promote the activity and performance of ADSCs in a three-dimensional space.

The exosomes with or without curcumin were resuspended in PBS and added to the adipogenic induction medium for ADSC culture. The curcumin-induced ADSC exosomes significantly promoted the number of adipocytes after adipogenic differentiation of ADSCs, but the volume of adipocytes was similar to that of the other two groups (Figure 4). This may indicate that after the exosomes are taken up by the ADSCs, the fat-forming ability of all cells is enhanced, whereas the cumulative ability of the lipids of single cells is not enhanced. This may be related to the promotion of cell activity by the exosomes, or could reflect the fact that lipid accumulation is already at an optimum under the premise that the total cell activity is not adjusted during the differentiation process.

Differential expression of PPARγ protein after adipogenic differentiation

The above experimental results indicated that curcumin-induced ADSC exosomes can promote the adipogenic differentiation of ADSCs. To further verify the expression of related proteins, we extracted the cell proteins after 14 days of adipogenic induction, which demonstrated that stem cell exosomes promoted the expression of PPARγ protein during adipogenic differentiation, which is a key protein regulating fat metabolism. This result demonstrated that exosomes increase the activity of ADSCs along with the protein content, and curcumin induces fat accumulation (Figure 5). This may indicate that the uptake of substances in the exosomes by ADSCs can increase the expression of related proteins and promote the accumulation of lipids throughout the adipogenic differentiation process.

Conclusion

To our knowledge, this is the first study to demonstrate that ADSC-derived exosomes can significantly promote the adipogenic differentiation of ADSCs, and curcumin treatment further enhanced the beneficial effects of ADSC exosomes. ADSCs are precursor cells of fat cells, which are indispensable in lipid metabolism. Therefore, our results highlight that ADSC exosomes likely play an important role in lipid metabolism.

Moreover, the content of PPARγ, the key protein of lipid metabolism [22], significantly increased within 2 weeks of adipogenic differentiation in the two groups treated with exosomes, and was further increased in the curcumin-enhanced exosome group compared with that of the control group, indicating a beneficial synergistic effect of curcumin and ADSC exosomes for the fat and protein accumulation of ADSCs.

However, there are some limitations of the study. For example, we did not identify the effects of exosomes and/or curcumin on specific pathways involved in adipose differentiation, and animal experiments will be required for verification of these results *in vivo*. Nevertheless, our results provide new insight into the fat metabolism mechanisms driving the accumulation of lipids in the body.

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