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Experimental Research on Astragalus Polysaccharides Intervening Angiogenesis of Human Adipose Derived Stem Cells

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Abstract

Objective: To preliminarily explore the mechanism of angiogenesis of human adipose-derived stem cells intervened by Astragalus Polysaccharides (APS).

Methods: Collagenase digestion and isolated culture of fat obtained from liposuction to obtained adipose derived stem cells (ADSC). P3 generation ADSC was stained with alizarin S and oil red O to confirm its osteogenesis and adipogenic differentiation. The cell surface differentiation antigen was detected by flow cytometry. Different concentrations of APS were employed to intervene ADSC, and the best concentration was screened through CCK-8 experiment. The co-culture system constructed by ADSC and human umbilical vein endothelial cells (HUVEC) was treated with APS at this concentration as the experimental group, and ADSC and APS alone as the control group to observe the changes of endothelial cell mobility. APS and ADSC intervention were termed as the experimental group, and ADSC intervention and no intervention as the control group to evaluate the formation rate of endothelial cell tubular structure. Expressions of Ang-1, Integrin and VEGF were detected by Western blot after 40 μg/mL APS intervened in ADSC for 48 hours; as well as the changes of ERK, p38, JNK expression in EC after 40 μg/mL APS intervention in ADSC.

Results: Stable amplified ADSC can be obtained by enzyme digestion. Alizarin red S staining and oil red O staining were all positive. The results of flow cytometry revealed positive CD90 (+) and negative CD34 (-) of P3 generation ADSC. Also, results of CCK-8 suggested that the optimal concentration of APS was 40 μg/mL. In the cell co-culture system, the mobility and tubular structure formation rate of EC after APS intervention in ADSC were significantly higher than those in the control group, and the difference was statistically significant ($P<0.05$). The expression of Ang-1, Integrin and VEGF was up-regulated after 48 hours of APS intervention in ADSC, and the difference was statistically significant ($P<0.05$). The expression of p-ERK1/2 and ERK1/2 was up-regulated in ADSC after 24 hours of APS intervention, and the difference was statistically significant ($P<0.05$); there was no significant difference in the expression of p38 and JNK.

Conclusions: Astragalus polysaccharide can promote the proliferation of ADSC and activate its function of promoting angiogenesis. The expression of such function may be related to the activation of Ang1-Tie2-ERK and/or VEGF-VEGFR-ERK signaling pathways.

Keywords: adipose derived stem cells; astragalus polysaccharide; angiogenesis

1. Introduction

Granular fat transplantation is an effective method for clinical treatment of tissue capacity deficiency induced by tumor, trauma and soft tissue degeneration.

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Early vascularization of transplanted fat is the key factor to improve its retention rate (1). Adipose derived stem cells (ADSCs) secrete a variety of cytokines that promote vascularization, which makes ADSC-assisted fat transplantation a research hotspot (2). Astragalus polysaccharides (APS) is the main active component of Astragalus membranaceus. Previous studies have shown that APS can relieve endothelial cell damage provoked by hypoxia and promote wound angiogenesis (3, 4). However, there still lacks of relevant research on whether APS can improve angiogenesis of transplanted fat and promote survival of transplanted fat. This study, through intervened ADSC in vitro by APS to investigate its role in promoting rapid vascularization and lay the foundation for further exploration of its mechanism.

2. Materials and Methods

2.1 Experimental Cells

Human adipose derived stem cells (ADSC) were extracted from discarded fat during human liposuction. Human umbilical vein endothelial cells (HUVEC) were purchased from GuangZhou Jennio Biotech Co., Ltd.

2.2 Main Reagents and Equipment

Astragalus Polysaccharide for Injection (Tianjin Cinorch Pharmaceutical Co., Ltd.). Type I collagenase (sigma). DMEM/F12 (Hyclone). Osteogenesis and adipogenesis induction medium (Cyagen Biosciences (Suzhou) Inc.). Oil Red O Staining Kit and Alizarin Red S Staining Kit (Jiangsu KeyGen BioTech Co., Ltd.). CD34, CD90 antibody (BD). CCK-8 Kit (Nanjing EnoGene Biotechnology Co., Ltd.). Matrix glue (Sigma). Ang-1, IntegrinαVβ3, VEGF, p-ERK1/2, ERK1/2, p-p38, p38, p-JNK antibody (CST).

Transwell culture plate (Corning). Flow cytometry (BD LSR-Ⅱ). Phase contrast inverted microscope (Olympus). Microplate scanning spectrophotometer (Bio-Rad). Electrophoresis and trans-blot apparatus (Bio-Rad). Laser detector (Odyssey).

2.3 Isolated Culture of Human ADSC

The fat mixture discarded by clinical abdominal liposuction was centrifuged at 1000 rpm for 3 minutes, and the upper granular fat layer was taken. After washing with PBS, and centrifuging at 1000 rpm, the upper fat was taken and added with 0.1% type I collagenase of equal volume, and digested in a 37 ℃ constant temperature rocking (100 rpm) for 1.5 hours. After 100 mesh sieving, the cell suspension was centrifuged at 1000 rpm for 5 minutes, and the cell precipitation was resuspended in DMEM/F12 medium containing 10% fetal bovine serum. After adjusting the cell density to 1×10^6/mL, the cell was inoculated in a 10 cm petri dish and placed in a 5% CO_2 saturated humidity incubator. The medium was changed 72 hours later, and then the solution was changed once every 2 days until 80% of the cells were fused for passage. The third passage (P3) cells were taken for subsequent experiments.

2.4 Phenotype Identification of Human ADSC

The third passage ADSC was digested and collected by 0.25% trypsin, washed twice with PBS and resuspended. The cell density was adjusted to 5×10^5/mL, and 100 μL of cell suspension was taken and placed in a flow tube. There were 20 L of PE-labeled CD34 and 20 L of FITC-labeled CD90 monoclonal antibodies were added to the cell suspension in turn, and the blank control group was not added with antibody. After mixing, the cell surface differentiation antigen was detected by flow cytometry after incubation at 4 ℃ for 30 minutes, protected from light.

2.5 Identification of Multidirectional Differentiation Function of Human ADSC

2.5.1 Adipogenic Induction Differentiation:

The third passage ADSC was inoculated into 6-well plates. When the cells fused to 80%, the medium was changed to 2 mL Adipogenic differentiation inducer (high glucose DMEM, 10% fetal bovine serum, 0.5 mmol/L IBMX, 1 μmol/L dexamethasone, 100 μmol/L indomethacin, 10 mg/L insulin), and the medium was changed every 3 days. After 2 weeks, oil red O staining was carried out following the Description of the kit, and observation and photographing were carried out under phase contrast inverted microscope.

2.5.2 Osteogenic Differentiation:

The third passage ADSC with satisfying growth was inoculated into 6-well plates. After the cells were fused to 80%, the cells were cultured with 2 mL of osteogenic inducer (low glucose DMEM, 10% fetal bovine serum, 10 mmol/L-sodium glycerophosphate, 0.1 μmol/L dexamethasone, 50 mg/L ascorbic acid). The inducer was changed every 3 days. After 4 weeks, alizarin red S staining was performed following the Description of the kit, and the cells were observed and photographed under phase contrast inverted microscope.

2.6 Detection of Cell Viability after Astragalus Polysaccharide Intervention in Human ADSC

The third passage ADSC with satisfying growth was taken, and the cell concentration was adjusted to 4×10^3/mL, and inoculated into 96-well plates. There were 6 groups of astragalus polysaccharide (10, 20, 40, 80, 160 μg/mL) with blank control and penta transfected in each group. After 12 hours of cell attachment, the cells were replaced with APS medium containing the corresponding concentration. After 24 hours of intervention, the samples were added according to the Description of CCK-8 kit and the optical density (OD) values of each well were measured by 450 nm microplate scanning spectrophotometer to calculate the cell viability of each group.

2.7 Effect of Astragalus Polysaccharide on Migration of Human ADSC to EC

The third passage ADSC with good growth were resuspended in DMEM/F12 containing 10% fetal bovine serum, and the cell concentration was adjusted to 1×10^5/mL and 500 μL were inoculated into the lower chamber of Transwell culture plate. After 24 hours, the amplified HUVEC was resuspended in the same medium; the cell inoculation density was adjusted to 2×10^4/mL and 200 μL was inoculated in Transwell upper chamber as control group 1. No ADSC was inoculated in the lower chamber, but HUVEC was inoculated in the upper chamber, and the medium containing 40 μg/mL APS as control group 2. ADSC was inoculated in the lower chamber, and HUVEC was inoculated in the upper chamber, and the medium contained 40 μg/mL APS as the observation group. After 24 hours, the upper chamber was taken out, and the non-migrating cells on the chamber surface were gently wiped off with cotton swabs. The migrated cells outside the chamber were stained with crystal violet solution containing 20% methanol at room temperature for 10 minutes. The number of migrating cells in each group was observed under microscope, and the relative mobility was calculated.

2.8 Detection of Tubular Structure Formation of EC after Intervention of Astragalus Polysaccharide on Human ADSC

The third passage ADSC with good growth were taken for resuspension, and the culture medium containing 10% fetal bovine serum DMEM/F12 and 40 μg/mL APS was grown for 24 hours, respectively, and the supernatant was taken for future use. Totally, 100 μL of Matrigel was taken and place in a 48-well plate pre-cooled at 4 ℃. and a 5% CO_2 incubator at 37 ℃ for 1 hour to solidify that matrigel. HUVEC was resuspended from ADSC culture supernatant without APS intervention as control group. HUVEC was resuspended in simple medium as blank control group. HUVEC was resuspended in conditioned medium after Astragalus polysaccharide intervened ADSC as observation group. The cell density was adjusted to 1×10^5/mL, and 200 μL was inoculated on the matrix gel surface and transfected in triplicate in each group. After 12 hours of culture, 3 fields of vision were randomly selected from each well, and photographed under a microscope. Image pro software was applied to calculate the sum ratio of lumen perimeter in unit field of vision.

2.9 Expression of Angiogenesis-related Proteins in Human ADSC after Astragalus Polysaccharide Intervention

The third passage ADSC was cultured in the medium containing 40 μg/mL APS after 24 hours of attachment. After 48 hours of intervention, the supernatant was washed with PBS, and RIPA was fully lysed on ice. After ultracentrifugation at 4 ℃, the supernatant was taken and the protein was quantified by means of BCA. In the other group, the third passage ADSC with good growth was taken, and the medium containing 40 μg/mL Astragalus polysaccharide was replaced after 24 hours of attachment, and the supernatant was taken for later use after 24 hours. Endothelial cells (ECs) were inoculated and attached to the wall for 24 hours and replaced with reserved supernatant. Proteins were collected and quantified following the above method after continuous culture for 24 hours. Followed by addition of loading buffer, it was heat denatured for 10 min; after protein separation by SDS-PAGE, PVDF membranes were transferred, BSA blocked, and primary antibodies (Ang-1, Integrin, VEGF, p-ERK1/2, ERK1/2, p-p38, p38, p-JNK) were incubated overnight at 4 °C, rinsed and fluorescently labeled secondary antibodies were incubated at room temperature for 1 h. After rinsing, laser scanners detected and calculated the band optical density values.

2.10 Statistical Analysis

SPSS 22.0 software was used for statistical analysis. Student t-test was used for the comparison of measurement data, and single-way ANOVA was used for the comparison between groups. $P < 0.05$ stands for the difference with statistically significant.

3. Result

3.1 *In Vitro* Expansion and Identification of Human ADSC

Relatively single ADSC can be obtained by collagenase type I digestion of granular fat obtained by clinical liposuction and repeated adherent culture *in vitro*. P3 ADSC grew adherently, with long spindle shape and excellent refractive index of cell membrane (see Fig. 1A-a). Flow cytometry detection of P3 ADSC surface differentiation antigen exhibited that the positive rate of CD90 was 94.71%, and the negative rate of CD34 was 99.81% (see Fig. 1B). Two weeks after ADSC adipogenic induction, oil red O staining revealed densely distributed various sizes oil droplets in cytoplasm, suggesting that stem cells differentiated into mature adipocytes and intracellular lipid synthesis was vigorous (see Fig. 1A-C). Four weeks after ADSC osteoblast induction, alizarin red S staining disclosed a large number of brown-red deep staining in the cell dense area, presenting that ADSC had a large amount of calcium salt deposition on its surface to form heterogeneous calcium nodules after differentiation into osteoblasts (Fig. 1A-D).

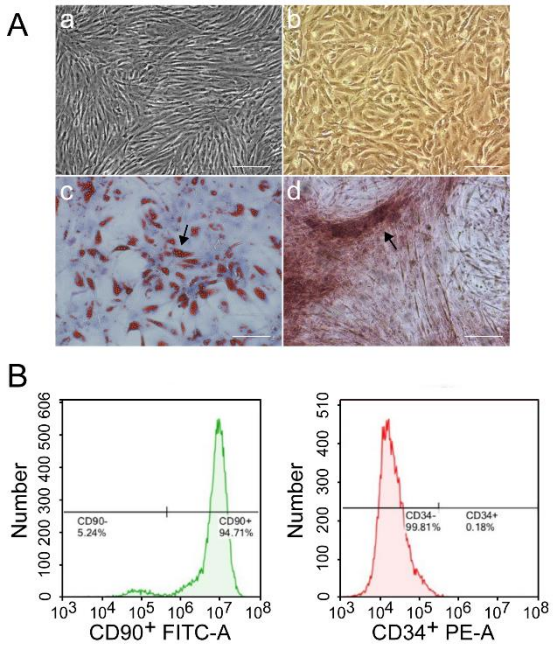

Fig 1 Differentiation function and differentiation antigen identification of ADSC A-a P3 generation; A-b HUVEC; A-c ADSC lipogenic differentiation, oil drop in cell at arrow; A-d ADSC osteogenic differentiation, calcium deposition at arrow; B flow cytometry detection of ADSC CD90 and CD34

3.2 Effect of Astragalus Polysaccharide on Proliferation Activity of Human ADSC

After ADSC were treated with different concentrations of APS for 24 hours, the CCK-8 assay results suggested that the OD values of cells in each group gradually increased with the uplift APS concentration, with the highest OD value in the 40 μg/mL group, which was significantly different compared with the control group (P<0.01). When APS intervention concentration continues to increase, the OD value of cells gradually decreases (see Fig. 2). Therefore, 40 μg/mL was confirmed as the best intervention concentration of APS.

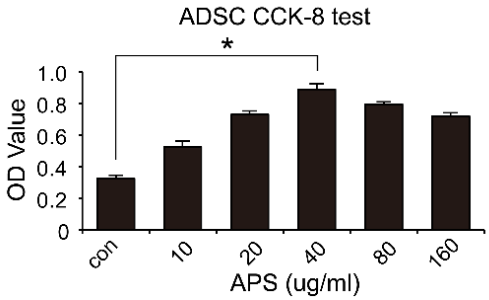

Fig 2 Cell proliferation after APS intervention in ADSC

3.3 Effect of Astragalus Polysaccharide on Endothelial Cell Migration and Tubular Structure Formation after Intervention of Human ADSC

A co-culture system of ADSC and HUVEC Transwell was established. With the extension of time, endothelial cells (EC) can migrate to different degrees outside the semipermeable membrane (see Fig. 3A). Compared with control group 1 (ADSC + EC), the cell mobility of control group 2 (APS + EC) was significantly increased at each time point, and the difference was statistically significant (P<0.05). Compared with the control group 2 (APS + ADSC + EC), the cell mobility in the observation group was also significantly increased at each time point, and the difference was statistically significant (P<0.05). Compared with each group, with the extension of culture time, the cell mobility also gradually increased, and the difference was statistically significant (P<0.05) (see Fig. 3 B).

Single HUVEC was inoculated on the surface of matrix glue. After 24 hours, scattered budded EC could be seen to proliferate with less complete lumen structure (see Fig. 3 D-a). In ADSC culture supernatant group, cell density increased and

lumen-like structures of different sizes were scattered in the field of vision (see Fig. 3 D-b). APS intervention ADSC conditioned medium group manifested higher cell density in visual field and more complete lumen-like structures of different sizes (see Fig. 3 D-c). The total perimeter of lumen per visual field in APS intervention group was significantly higher than that in ADSC group and the control group (P<0.05) (see Fig. 3 C).

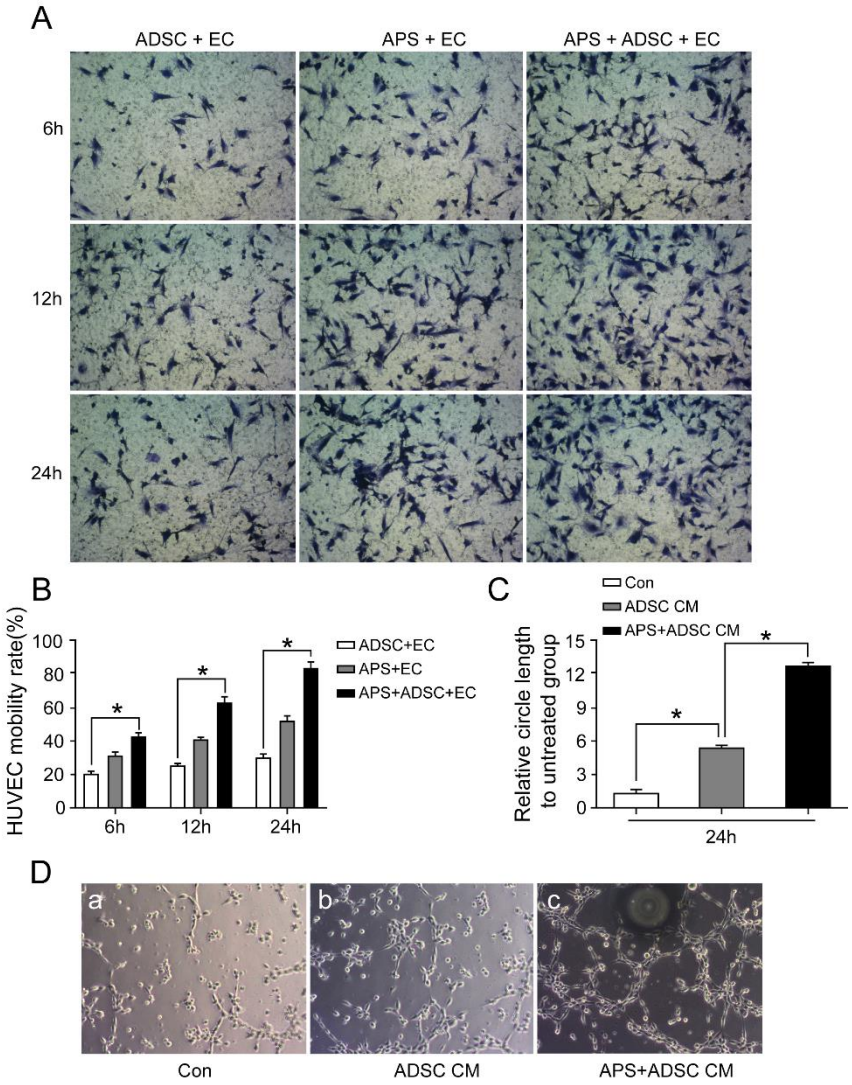

Fig 3 The effect of APS and ADSC on endothelial cell migration and tubular structure formation was significantly higher in group APS + ADSC + EC than in the control group (P < 0.05); the relative perimeter of endothelial cell tubular structure in group APS + ADSC was significantly greater than that in the control group (P < 0.05)

3.4 Changes of Expression of Angiogenesis-related Proteins after Astragalus Polysaccharide Interfering with ADSC

The optimal concentration of 40 μg/mL APS was used to treat ADSC for 48 hours. Cell proteins were collected and the expressions of Ang-1, Integrin and VEGF were detected by WesternBlot. The results showed that the expression level of Ang-1 increased gradually, and the difference of expression level at each time point was statistically significant (P<0.05) (see Figs. 4 A, C). The expression of VEGF increased gradually, and the expression of VEGF did not increase significantly at 12 hours, but the difference was statistically significant at 24 hours (P<0.05) (see Figs. 4 A, D). The expression of Integrin gradually increased with time, with the peak value at 24 hours,

and then began to decrease, but it was still significantly higher than that before the intervention (P<0.05) (see Figs. 4 A, B). After 24 hours of APS (40 μg/mL) intervention of ADSC; MAPK downstream related protein expression (see Fig. 5 A), with time prolonged, p-ERK1/2 and ERK1/2 expression gradually increased, p-ERK/ERK expression was higher than the control group at each time point, and the expression increased most significantly at 24 hours, and the difference was statistically significant (P<0.05), (see Fig. 5 B).

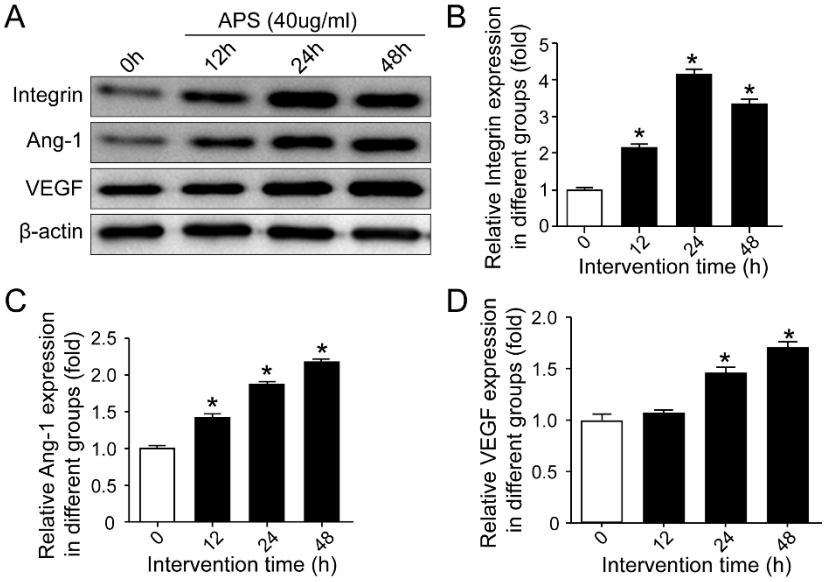

Fig 4 After APS intervention for 48 hours, the expression of Ang-1 and VEGF gradually increased (P < 0.05). The peak of integrin expression was 24 hours after intervention, the difference was statistically significant (P < 0.05)

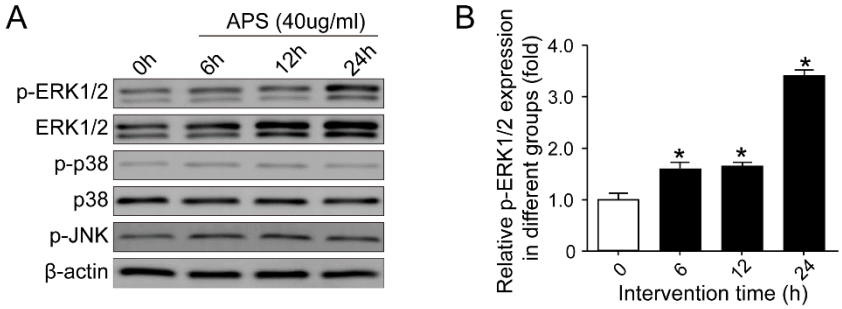

Fig 5 After 24 hours of APS and ADSC intervention, the expression of MAPK pathway related proteins in endothelial cells gradually increased in p-ERK1/2 and ERK1/2 (P < 0.05). There was no significant difference in p38 and JNK expression

4. Discussion

Free fat transplantation is an important method for clinical treatment of soft tissue capacity loss due to trauma, tumor, malformation and tissue degeneration. However, the rapid absorption of transplanted fat seriously affects the long-term efficacy of the operation. A large number of studies have shown that early rapid vascularization of transplanted fat is the key to improve its survival rate [(1)]. Adipose-derived stem cells contained in adipose tissue can not only differentiate into adipocytes under appropriate environment, supplement the number of adipocytes, more importantly, they also secrete a variety of cytokines

that promote vascularization and angiogenesis, which can promote rapid vascularization around transplanted fat particles [5], thus improving the survival rate of fat and inhibiting its excessive liquefaction and absorption. Therefore, mixed transplantation of granular fat and adipose-derived stem cells or stromal vascular fragment (SVF), namely, ADSC-assisted fat transplantation, becomes an important means to improve the survival rate of transplanted fat [6].

Collagenase digestion *in vitro* is a classical method to obtain relatively single ADSC. Adipogenic, osteogenic, chondrogenic and other multidirectional differentiation and the expression of relatively specific cell surface differentiation antigens are the main identification elements of ADSC [7]. Adipose-derived stem cells obtained in this experiment can clearly differentiate into osteoblasts and adipocytes after appropriate induction. At the same time, flow cytometry revealed that the cells expressed CD90, which was highly expressed in mesenchymal cells, and CD34, which was highly expressed in hematopoietic cells, were in line with the current consensus on ADSC expressing differentiation antigen [6]. Although SVF-assisted fat transplantation is more conducive to fat survival, and obtaining SVF through physical methods can effectively avoid ethical problems, it will be more difficult to explore its mechanism of action due to the complex composition of SVF. Therefore, ADSC, the main component of SVF, was still selected for *in vitro* cell experiments.

Astragalus membranaceus is widely used in clinical traditional Chinese medicine compound due to its effects of promoting blood circulation, removing blood stasis, invigorating the middle warmer and invigorating qi. Astragalus polysaccharide is the main active ingredient of Astragalus membranaceus, which is mainly composed of hexuronic acid, glucose, fructose, rhamnose, arabinose, galacturonic acid, *etc.* [8, 9]. Modern medicine has proved that Astragalus polysaccharide embraces many functions such as immune regulation, inhibition of inflammation, antioxidant stress, promotion of tissue regeneration, *etc.* [8, 10]. Studies in recent years have shown that astragalus polysaccharide can accelerate the healing of ischemic ulcer wounds. On the one hand, Astragalus polysaccharide can promote the homing, proliferation and differentiation of bone marrow stromal stem cells to the wound surface, on the other hand, it promots the proliferation and functional expression of wound EC and accelerated local angiogenesis [3, 10]. In this study, gradient concentrations of APS were used to intervene ADSC following the previous experimental basis, and the viability effect of APS on ADSC was confirmed to be bidirectional by CCK-8 cell viability assay. When APS concentration is lower than 40 μg/mL, its intervention concentration is positively correlated with ADSC activity. When APS concentration is higher than 40 μg/mL, its intervention concentration is negatively correlated with ADSC activity. Finally, 40 μ μg/mL was determined as the best intervention concentration of APS, which was basically consistent with the literature report [8, 11].

Previous studies have shown that APS can promote the proliferation and migration of vascular EC to initiate rapid vascularization [11]. Our experimental results are consistent with this (see Fig. 3 A). Compared with ADSC and EC co-culture group and APS intervention EC group, the mobility of EC in APS + ADSC + EC group increased significantly. At the same time, the cell mobility of APS intervention EC group is significantly higher than that of ADSC and EC co-culture group, which suggests that APS can not only directly act on EC, but also indirectly promote EC migration through ADSC. The migration of EC to wound surface or tissue transplantation area is a prerequisite for angiogenesis, involving the regulation of complex signal pathways mediated by various chemokines. Some studies have shown that perivascular cells such as pericytes, macrophages and bone marrow mesenchymal stem cells can up-regulate Ang-1 expression under hypoxia and injury environment, and regulate downstream microtubulin expression through paracrine effect binding to Tie-2 receptor on endothelial cell surface, thus promoting endothelial cell migration [12-14]. This experiment suggests that Ang-1 expression in ADSC is significantly up-regulated within 48 hours after APS intervention and has a time-dependent relationship (see Figs. 4 A, C), which is consistent with the literature.

EC migrating to the microvascular defect need to proliferate continuously and form lumen-like structures and communicate with the microvascular broken end of the recipient region to exert the function of rapid vascularization. Matrigel holds a certain 3-D structure, which is conducive to the formation of tubular structure of EC inoculated on the surface and is convenient to evaluate the effect of exogenous intervention [15]. As shown in Fig. 3D, the effect of ADSC conditioned medium after APS

intervention on promoting the formation of tubular structure of vascular EC is significantly higher than that of ADSC conditioned medium group without APS intervention, suggesting that the function of ADSC on promoting angiogenesis is enhanced after APS intervention, and the cytokines secreted by ADSC are helpful for the tubular proliferation of EC. Literature reported that a variety of signal molecules participate in EC angiogenesis. Secretory Ang-1 binded to endothelial cell Tie-2 receptor and activated downstream MAPK signaling pathway through receptor tyrosine kinase to promote endothelial cell proliferation and expression of angiogenesis-related proteins [(16)]. There are three classical pathways in MAPK signaling pathway: ERK1/2, p38 and JNK. The results of this study disclose that after ECs were cultured in ADSC conditioned medium after APS intervention for 24 hours, the expression of ERK1/2 protein and its phosphorylated active form in EC was significantly up-regulated, while the expression of p38 and JNK did not significantly change (see Figs. 5A and B), suggesting that Ang-1 secreted by ADSC may play a role in promoting angiogenesis through ERK signaling pathway. As shown in Figs. 4 A, D, the expression of VEGF in ADSC after APS intervention is gradually up-regulated. Some studies have shown that the binding of VEGF to VEGFR2 receptor on the surface of EC can also activate MAPK-ERK signaling pathway to promote the proliferation of EC and the shaping of neovascularization [(14)]. In addition, this study found that the expression of integrin in ADSC after APS intervention was significantly up-regulated (see Figs. 4A and B). Previous studies have shown that the expression of integrin in perivascular cell membrane is conducive to its anchoring with extracellular matrix and to maintaining its continuous function of promoting vascularization (13). At the same time, Integrin αVβ3 of cytoplasmic cells can combine with VCAM-1 molecules on the surface of EC and vascular smooth muscle cells, which can promote the coordination of various cell biological behaviors during angiogenesis [(17)].

To sum up, Astragalus polysaccharide promotes the proliferation of ADSC and activates its function of promoting angiogenesis. The expression of this function may be related to the activation of Ang1-Tie2-ERK and/or VEGF-VEGFR-ERK signaling pathways, which is worthy of further in-depth study.

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