An In Vitro Study of the Effects of Mechanical and Enzymatic Isolation of Stromal Vascular Fraction on Wound Healing

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Abstract: The adipose-derived stromal vascular fraction (SVF) is considered to be an attractive source of stem cells in cell therapy. Besides stem cells, it also contains functional cells, such as macrophages, precursor cells, somatic stem cells, and pericytes. Collagenase digestion is the most frequently used method to isolate SVF, but it is time-consuming and costly and has some problems, such as infectious agents and immune reactions. In this research, we compared the yield, cell population ratios, and cell viability when isolating SVF by the ultrasonic physics (U-SVF) method and traditional enzymatic method (E-SVF). Then, we isolated exosomes from U-SVF and E-SVF, respectively, and cocultured them with fibroblasts to investigate the potential of applying this cell secretion in wound repair. The results showed that there was no significant difference between the ultrasonic method and enzymatic method in terms of cell viability, cell numbers, or the expression of CD markers of stem cells. However, exosome analysis identified a greater number and smaller size of exosome particles obtained by U-SVF. In terms of cell proliferation efficiency, although the proliferation efficiency of U-SVF was lower than that of E-SVF. Trilineage differentiation experiments revealed that both E-SVF and U-SVF had good differentiation ability, owing to high stem cell content. Finally, E-SVF and U-SVF exosomes were cocultured with fibroblasts. The efficiency of fibroblast migration increased in the SVF exosome treated groups, and the expression of related genes (integrin $\alpha 5\beta 1$) was slightly upregulated; however, the expression of FAK, AKT, ERK, and RhoA was significantly upregulated at 24 hours. From the abovementioned experiments, we found that there was no significant difference in stem cell-related characteristics between SVF isolated by ultrasonic cavitation and SVF isolated by the enzymatic method. In addition, exosomes secreted by SVF may have excellent therapeutic effect on skin injuries, which provides a new viewpoint and therapeutic strategy for soft tissue repair.

Key Words: adipose-derived stromal vascular fraction, ultrasonic physics method, exosome

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In the past few decades, biological therapy has been gradually replacing traditional medicine or surgical therapy to treat various diseases and injuries. Adipose tissue is considered to be an important source of biological products for the treatment of chronic and acute degenerative diseases.[1] Adipose tissue contains stromal vascular fraction (SVF) and adipocytes. Stromal vascular fraction consists of a heterogeneous population of stem and stromal cells, including stem cells, progenitor cells, endothelial cells (ECs), monocyte/macrophages, smooth muscle cells, and pericytes.[2–5] The present study reported that the concentration of adipose-derived mesenchymal stem cells (MSCs) is ~500 times higher than that of bone marrow-derived MSCs.[3] Tissue engineering involving SVF has now become the focus of stem cell research, regenerative medicine, minor transplantation, and treatment of degenerative, congenital, or traumatic diseases and bone, joint and soft tissue defects, and it has important clinical significance.[6–8]

As SVF is composed of heterogeneous cell groups, it can be used to achieve good therapeutic results in immune regulation, anti-inflammation, and angiogenesis.[9,10] Among them, SVF cell populations contain a large number of stem cells, which can be induced to differentiate into cartilage, bone, and adipocytes, which can be applied in the treatment of various diseases, including multiple sclerosis, peripheral neuropathy, osteoarthritis, and diabetic foot ulcer[11] At present, an increasing number of studies have shown that the regeneration and repair of tissues are mainly achieved through direct intercellular contact and secretome-based paracrine activity, such as exosomes, growth factors, miRNA, mRNA, and proteins.[12–14] Through paracrine activity using the culture medium of human Wharton jelly-derived MSCs, upregulation of genes related to reepithelialization (*transforming growth factor-β2*), neovascularization (*hypoxia-inducible factor-1α*), and fibroproliferation (*plasminogen activator inhibitor-1*) of fibroblasts could be promoted and the proliferation and migration efficiency of fibroblasts could be accelerated; when the cell culture medium was directly used in the nude mouse wound healing model, it could accelerate the wound healing rate.[15] In addition, when exosomes secreted by human umbilical cord MSCs were applied to treat skin burns in rats, it was found that exosome-treated wounds exhibited significantly accelerated in vivo epithelial regeneration and wound healing, and the expression levels of Cytokeratin 19, proliferating cell nuclear antigen and collagen I increased.[16] Therefore, SVF cell populations or their secretions have great development potential in clinical treatment.

Stromal vascular fraction can be obtained from adipose tissue by enzymatic or nonenzymatic methods,[17] and the most commonly used isolation technique is collagenase digestion of adipose tissue.[18] A large number of nucleated cells could be obtained by enzyme digestion (approximately 100,000–1,300,000 nucleated cells per gram of lipoaspirate), and the cell viability exceeded 80%.[19] However, the enzymatic isolation method involves the use of xenogenic components, which may expose cells to infectious agents and immune reactions.[20] In addition, it is very time-consuming (requiring reaction at 37°C for more than 1 hour) and costly (costs of $2–$5 per gram of tissue processed using GMP grade enzymes).[5] Moreover, enzyme digestion can destroy the functional extracellular matrix.[21] The complex operation process of enzyme digestion has brought many obstacles to the establishment of good manufacturing practice. Therefore, for the convenience of clinical practice, we have developed a mechanical isolation method using physical force to destroy tissues and isolate cells from the adipose matrix, that is, shaking, vortexing, ultrasonic cavitation, ultrasharp blade systems, and centrifugation.[5,22–26] The cell yield obtained by the mechanical method ranged from 10,000 nucleated cells/mL lipoaspirate to 240,000 nucleated cells/mL lipoaspirate, and the cell viability was approximately 90%.[19] The advantages of this method are that the treatment requires less time and is simpler than that of the enzymatic method, there is no contact with reagents, there is no difference in composition among batches of consumables or reagents, the cell yield is similar to that of the enzymatic method, cells without

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xenogenic contamination can be obtained in a short time (30–90 minutes), and it can be used immediately in cosmetic or plastic surgery.

Based on the aforementioned fact that SVF can be easily isolated from adipose tissue in large quantities and has great potential for application in treatment, as well as the advantages of isolating SVF by physical methods, many organizations in regenerative medicine have begun to study the isolation of SVF by physical methods. In this study, we tried isolating SVF from adipose tissue using the ultrasonic cavitation (U-SVF) method and traditional enzymatic method (E-SVF) and compared SVF yield, characteristic proteins, differentiation ability, and exosome content to understand the cell population composition ratios and differentiation potential of SVF obtained by different methods. Finally, fibroblasts were combined with exosomes secreted by U-SVF or E-SVF to better understand the possibility of applying exosomes secreted by SVF in wound healing research.

MATERIALS AND METHODS

Isolation of SVF by Physical and Enzymatic Methods

In this research project, adipose tissue was obtained from humans according to the human trial practice of Tri-Service General Hospital (IRB 2-107-05-147). The donors were aged 20 to 60 years. Samples obtained by adipectomy or liposuction were used. The samples from at least 3 different donors were used to confirm experimental reproducibility. The adipose tissue was divided into 2 aliquots for isolation of SVF by the mechanical and enzymatic methods.

The E-SVF method[27] can be briefly described as follows: soak 50-mL adipose tissue in 0.1% collagenase/L-15 medium for reaction for 24 hours, and centrifuge at 1500 rpm for 5 minutes. After removing the supernatant, the precipitate consists of E-SVF.

The physical isolation method (U-SVF) is to use a BeStem cell processing platform based on the ultrasonic cell recovery unit (StroMed; Cell-Innovations, Australia) for isolation under aseptic conditions, and the method of usage is as stated in the literature,[25] which can be briefly described as follows: centrifuge 50-mL adipose tissue at 200*g* for 5 minutes, and then remove blood and oil. Treat with ultrasonic cell recovery unit for 5 minutes, centrifuge again at 800*g* for 5 minutes to remove oil, add normal saline containing sodium bicarbonate to constitute 45 mL, shake and mix evenly, and centrifuge again at 800*g* for 5 minutes. The precipitate is U-SVF.

Cell Viability

The cell survival rates (the proportion of nonred fluorescent cells to the total number of cells) of SVF isolated by the mechanical method or enzymatic method were detected by flow cytometry. The precipitated cells were dissolved in 1 mL phosphate-buffered saline (PBS) and 1 μL propidium iodide was added to test the cell survival rate.

Cell Proliferation Rate and Cell Morphology

The SVF cell populations isolated by the enzymatic and physical method were grown under the same cell culture conditions. Low glucose DMEM/F12 (Gibco Thermo Fisher Scientific) and EBM-2 culture medium (LONZA, Switzerland) were mixed and supplemented with 20% fetal bovine serum and 1% antibiotics (Gibco Thermo Fisher Scientific) for culture (LD/F12/EGM-2). Cultured cells at passage 2 were used. To analyze the cell proliferation rate, the cell populations obtained by the 2 different methods were seeded in 24-well tissue culture polystyrene at a concentration of 1×10^4 cells/well. On days 0, 7, and 14, cell proliferation analysis was conducted using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, USA). The reagent and culture medium were mixed at a ratio of 1:9, and 300 μL was added to each well for reaction at 37°C for 4 hours. The absorbance at 570 nm was measured using an ELISA assay plate reader, and the cell proliferation analysis was calculated by the calibration curve method. Cell morphology was observed under a microscope on days 7 and 14.

Analysis of Cell Surface Antigens by Flow Cytometry

The cells were evenly dispersed with 5% bovine serum albumin/PBS solution, placed at 4°C for approximately 1 hour, and then mixed with 2 μL of CD73, CD90, CD105, CD45, CD34, and CD31 antibodies (BioLegend, USA) for staining. After 1 hour, the cells were centrifuged at 1500 rpm for 5 minutes, the supernatant was removed, and the cells were washed twice with PBS. The cells, at passages 0 and 2, were suspended in 1 mL Hank balanced salt solution and placed on ice protected from light to be used for relevant analysis by flow cytometry (BD, USA).

Isolation and Quantification of Exosomes

The exosomes of SVF obtained by different isolation methods were extracted using ExoQuick-TC Precipitation Solution (System Biosciences, CA) at passage 2. The steps are briefly as follows: centrifuge at 3000*g* for 15 minutes to remove cells and cell fragments; transfer the supernatant to a sterile container; add the proper amount of ExoQuick-TC; flip over the test tube to mix the liquid evenly; react at 4°C for 24 hours; and centrifuge the ExoQuick-TC/cell solution mixture at 1500*g* for 30 minutes. After centrifugation, remove the supernatant and resuspend the precipitated exosomes in 100 to 500 μL buffer. Finally, analyze the exosome characteristic proteins CD9, CD63, and CD81 by flow cytometry. Dilute the precipitated and centrifuged exosomes with pure water, inject the samples into the inspection tank with a syringe, and then put the inspection tank into the NanoSight NS3000 (Malvern Panalytical) for the machine to automatically scan and analyze particle size distribution range and concentration of exosomes, as well as to record the analysis results.

Isolation Fibroblast Migration Assay

First, fibroblasts were isolated.[28] The samples were obtained from human male prepuce obtained by circumcision, according to the human trial practice of Tri-Service General Hospital (IRB 2-107-05-147). The method for isolation is as follows: soak approximately 5 cm × 4 cm of prepuce tissue in 0.1% Dispase II/L-15 solution and react for 24 hours at room temperature; then, use a blade to isolate the dermis from the epidermis, immerse the dermis in 0.1% collagenase to react for 48 hours, centrifuge at 1500 rpm for 5 minutes, remove the supernatant, and add 1 mL fresh culture medium to resuspend the precipitated cells. After the human fibroblasts were isolated from prepuce tissue, they were cultured in high glucose DMEM supplemented with 10% fetal bovine serum. The culture medium was changed every 2 days, and cells from passages 3 to 5 were used in the experiment.

Migration Assay

The fibroblasts were seeded in a 24-well cell culture plate at 1×10^4 cells/well. After the cells adhered to the plate surface, the original culture medium was removed, and the exosomes (200 μg/mL) isolated from SVF obtained by different isolation methods were added for coculture. After 3 days, the cells were harvested with 0.25% trypsin. Then, 1×10^4 fibroblasts in 1 mL LD/F12/EGM-2 medium were seeded into the upper chamber of transwell inserts (8 μm pore; Millipore) and then incubated at 37°C for 24 hours. After culturing for 24 hours at 37°C, cells in the upper chamber were removed. The number of fibroblasts that migrated to the other side of the transwell inserts was observed using a microscope.

Wound Healing Assay

The effects of the E-SVF exosomes and U-SVF exosomes on fibroblast migration were evaluated by scratch assay. Fibroblasts were seeded in 6-well cell culture plates at a concentration of 10^5 cells/well. After culturing for 2 days, the confluent cell monolayer was scratched

using a sterile cell scraper (BD Falcon). The samples were then washed and the edge of the scratch was smoothed with PBS. Therefore, fresh medium containing E-SVF exosomes or U-SVF exosomes (800 μg/mL) was added for coculture. Images were recorded at 0, 12, and 24 hours after the monolayers were scratched.

Expression Analysis of Genes Related to Cell Differentiation Ability

The gene expression levels were analyzed by real-time polymerase chain reaction (PCR). The expression levels of Runx2, Sox9, PPARγ2, and GADPH genes in cell populations isolated by different isolation methods were analyzed after culture for 7 and 14 days, and the expression levels of integrin (ITG) αv, ITGα5, ITGβ1, ITGβ3, FAK, RhoA, Akt, ERK, and β-actin genes isolated from SVF obtained by different isolation methods after culture for 24 hours were analyzed. The primer sequences are shown in Table 1. After the cells were dispersed with 0.25% trypsin, the cells were collected by centrifugation. RNA was extracted according to the instructions for RNase-Free DNase I Set (Geneaid, Taiwan), and 1 mL Trizol (Invitrogen, USA) was added for reaction for 10 minutes. Subsequently, the RNA was added with 1 mL absolute ethanol, mixed evenly, and put into the RB column for centrifugation at 15,000*g* for 1 minute to remove the liquid. Then, it was mixed with 400 μL wash buffer and centrifuged at 15,000*g* for 1 minute to remove the liquid. After this, 50 μL DNase I/reaction buffer was added for reaction at room temperature for 15 minutes, 400 μL prewash buffer was added for centrifugation at 15,000*g* for 1 minute, and lastly, a 600-μL wash buffer was added for centrifugation at 15,000*g* for 1 minute to remove the liquid; finally, 30 μL RNase-free water was added to the RB column for centrifugation at 15,000*g* for 2 minutes, and RNA was collected. Then, cDNA was synthesized using SunScript reverse transcriptase (Bio-Genesis Technologies, Taiwan) and amplified by PCR. All RNA samples were reacted at 42°C for 60 minutes and at 72°C for 10 minutes to prepare cDNA. Finally, reaction was carried out by real-time PCR (Roche, Switzerland) under the conditions of 30 seconds at 94°C, 30 seconds at 62°C, and 50 seconds at 72°C, for a total of 30 cycles. The gene expression levels of ITGαv, ITGα5, ITGβ1, ITGβ3, FAK, RhoA, Akt, and ERK were normalized to the expression of β-actin at 24 hours. Furthermore, the gene expression levels of Runx2, PPARγ2, and Sox9 were normalized to that of GAPDH. The gene expression levels of Runx2, PPARγ2, and Sox9 in differentiated SVF were relative to those in undifferentiated SVF on days 7 and 14.

TABLE 1. Primers Used in Real-Time PCR

Gene	Sense Primer	Primer Annealing Temperature (°C)
Runx2	CAGCGTCAACACCATCATTC	62
	CAGACCAGCAGCACTCCATA	
PPARγ2	TCTACTCCACATTACGAAGACA	62
	ACTCCATAGTGAAATCCAGAAG	
Sox9	CTAAGAGGCATCCAAACAACACA	62
	CCCTCGCTGCTAAAGTGTAATAA	
ITG αv	GGTCCATTCTGCATTGTATT	62
	ATTTTCTGTCTCACCCAATG	
ITG α5	GTGGGCCAACAAAGAACACT	62
	TGAGTTCTGATTCCCCTTGG	
ITG β1	ATCCCAGAGGCTCCAAAGAT	62
	CCCCTGATCTTAATCGCAAA	
ITG β3	GTTTTTAGTTGGGAGATCTGAG	62
	CTACATCAGGAGAGACGTAACTATT	
FAK	CTTTGAGATCCTGTCTCCAGTCTAC	62
	CTTGTCCGTTAGGTAACTGATTCC	
RhoA	TATCGAGGTGGATGGAAAGC	62
	TTCTGGGGTCCACTTTTCTG	
Akt	ACTCATTCCAGACCCACGAC	60
	AGCCCGAAGTCCGTTATCTT	
ERK	AATCACACGGTAGACACTGAAATGCC	60
	CATCATCCCATCTAAAATGTCCCCTG	
GADPH	AACCTGCCAAATATGATGAC	62
	ATACCAGGAAATGAGCTTGA	
β-actin	GGACTATGACTTAGTTGCGTTAC	62
	TTTGCATTACATAATTTACACGA	

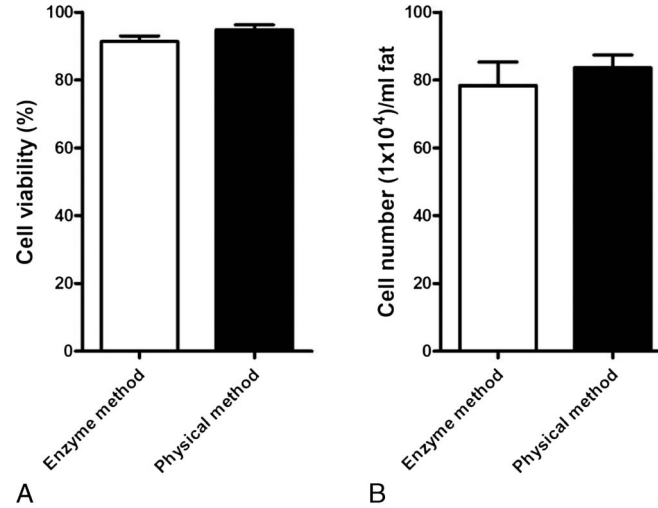

FIGURE 1. (A) Cell viability and (B) cell number of SVF isolated using enzyme and nonenzyme (physical) method.

Statistical Analysis

Data are expressed as mean ± standard error of the mean. All in vitro experiments used 3 independent repetitions, and cells from at least 3 different donors were used to confirm experimental reproducibility. Statistically significant differences were analyzed by one-way analysis of variance. Differences were considered significant at a P less than 0.05.

RESULTS

Cell Viability and Cell Count

The numbers and viability of cells isolated from adipose tissue by the enzymatic and ultrasonic physical methods are shown in Figure 1. The viability of E-SVF cells was approximately 90%, whereas that of U-SVF cells was slightly higher, at approximately 95%, with no significant difference between them. For the number of isolated cells, approximately 80×10^4 cells/mL could be isolated from E-SVF, whereas the total number of isolated nucleated U-SVF cells was approximately 85×10^4 cells/mL fat. There was no significant difference in cell viability and cell number between the 2 SVF isolation methods.

E-SVF and U-SVF Characteristics

Characteristic proteins on the cell surface of SVF isolated from adipose tissue by the enzymatic method and ultrasonic physical method at passages 0 and 2 were analyzed by flow cytometry (Fig. 2A), and the quantitative results are shown in Figures 2B and 2C. By comparing the expression of adipose-derived stem cell (ADSC) and hematopoietic cell

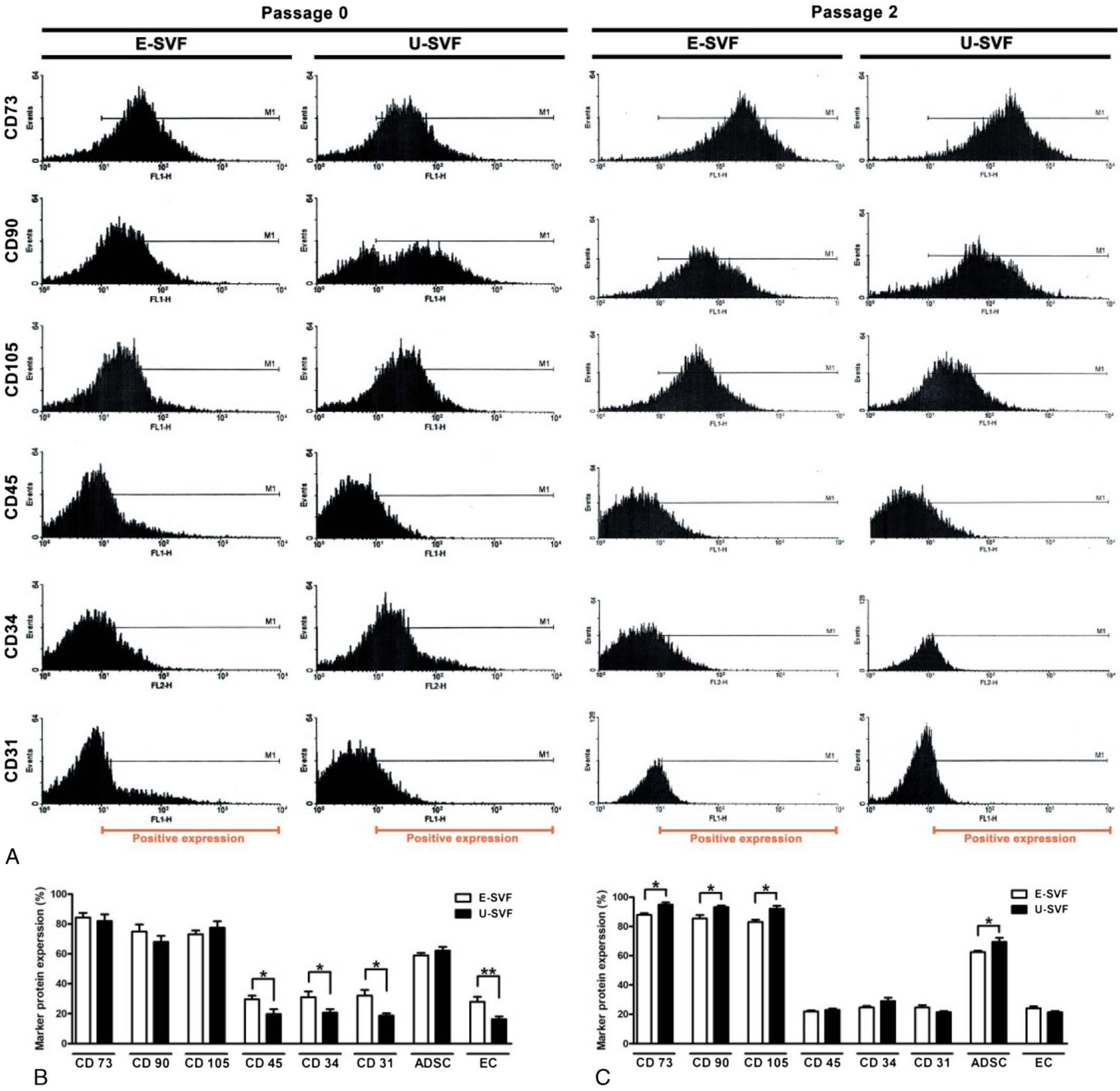

FIGURE 2. Characterization by flow cytometry of ADSCs and ECs of E-SVF and U-SVF at (A) passage 0 and (B) passage 2. The quantitative data of cells at (C) passage 0 and (D) passage 2. ADSCs: CD 73^+/CD 90^+/CD 105^+/CD 45^-. ECs: $CD34^+/CD31^+$. $*P < 0.05$; $**P < 0.01$.

CD markers in U-SVF and E-SVF at passage 0, it was found that the expression levels of CD73 (~80%), CD90 (~70%), and CD105 (~75%) were not significantly different between U-SVF and E-SVF, but the expression levels of E-SVF CD45, CD34, and CD31 were approximately 1.5 to 2 times those of U-SVF, with significant difference. The expression levels of E-SVF and U-SVF were similar when analyzing the known combination of ADSC CD marker ($CD73^+/CD90^+/CD105^+/CD45^-$). However, when analyzing the hematopoietic cell marker combination ($CD34^+/CD31^+$), it was found that the expression of E-SVF was twice more than that of U-SVF. At passage 2, the expression level of the ADSC CD marker combination in U-SVF was higher than that of the hematopoietic cell marker combination in E-SVF.

Cell Proliferation Test and Morphology

Figure 3A shows the cell morphology of E-SVF and U-SVF cultured for 7 and 14 days. Most cells were adhered to the culture plate in a spindle shape, and a small portion of cells were adhered to the culture plate in round shape. When cultured for 7 days, the number of cells in E-SVF was more than that in U-SVF under the microscope. According

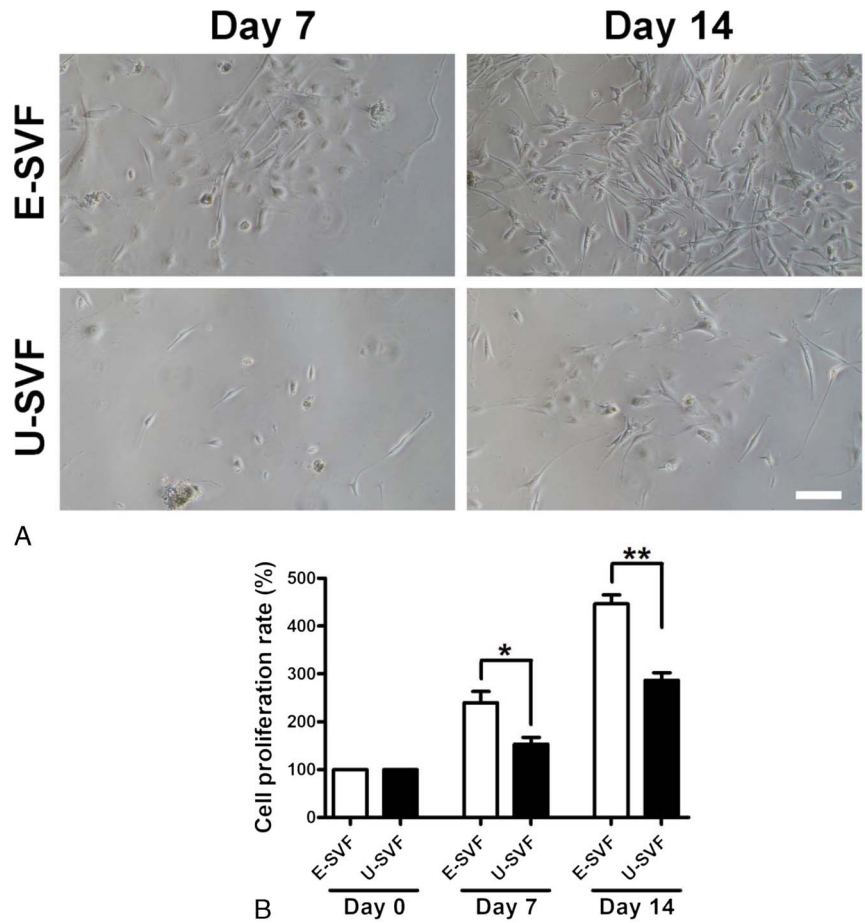

FIGURE 3. (A) Morphology of E-SVF and U-SVF cultured on tissue culture polystyrene at days 7 and 14. The scale bar represents 250 μm. (B) Cell proliferation rate was expressed as the percentage and evaluated by MTT assay at days 0, 7, and 14. $*P < 0.05$; $**P < 0.01$.

to the MTT assay (Fig. 3B), the cell proliferation efficiency of E-SVF (~250%) was faster than that of U-SVF (~150%). At 14 days, although the proliferation efficiency of E-SVF (~450%) was still higher than that of U-SVF (~300%), it was found that the cells in both groups continued to proliferate stably, which confirmed that the culture medium (LD/F12/EGM-2) used in this study could be used to maintain the proliferation of SVF.

Analysis of Cartilage, Bone, and Adipose Differentiation Ability

Trilineage differentiation experiments were performed to assess the multipotency of the E-SVF and U-SVF. The expression levels of genes in the bone (Runx2), adipose (PPARγ2), and cartilage (Sox9) of E-SVF and U-SVF were displayed as those in differentiated SVF vs. undifferentiated SVF, and the results are shown in Figure 4. The gene expression results of bone (Runx2), adipose (PPARγ2), and cartilage (Sox9) showed that the differentiation ability of U-SVF was better than that of E-SVF. These results indicated that U-SVF and E-SVF had MSC characteristics and pluripotency.

Exosome Characteristics

The analysis results of exosomes from U-SVF and E-SVF are shown in Table 2. The particle size of E-SVF (~125 nm) was slightly larger than that of U-SVF (~110 nm). The number of particles in U-SVF (~10×10^{11} particles/mL) was greater than that in E-SVF (6×10^{11} particles/mL). U-SVF and E-SVF both expressed exosomal markers, such as CD9, CD63, and CD81 proteins. The results indicated that we successfully isolated exosomes from SVF, which were consistent with the exosomes defined.

Cell Migration

The results from the scratch closure test are displayed in Figure 5A. The migration of fibroblasts increased after 12 h and 24 h in the presence of exosomes compared to the migration in the untreated group (fibroblast). For the transwell migration assay, the number of fibroblasts that migrated from the upper well to the lower well of a transwell after 24 hours is shown in Figure 5B. In all groups, the average number of fibroblasts in the untreated group (fibroblast; ~15 cells/field) was lower than that in the SVF exosome-treated fibroblast group (fibroblast+U-SVF: ~25 cells/field and fibroblast+E-SVF: ~20 cells/field) in the lower well. There was no significant difference in the number of migrated fibroblasts between the fibroblast+E-SVF and fibroblast+U-SVF groups. These results suggest that the number of migrated fibroblasts might be enhanced by treatment with SVF exosomes.

Migration Gene Expression

The expression of integrin genes related to cell migration was analyzed, and the results are shown in Figures 6A–H. The expression levels of ITGβ3 gene in the fibroblast+E-SVF and fibroblast+U-SVF groups were lower than those in the fibroblast group. The expression of ITGβ1, ITGα5, and ITGαv genes showed the same trend in all groups. The gene expression of integrins, FAK, RhoA, Akt, and ERK

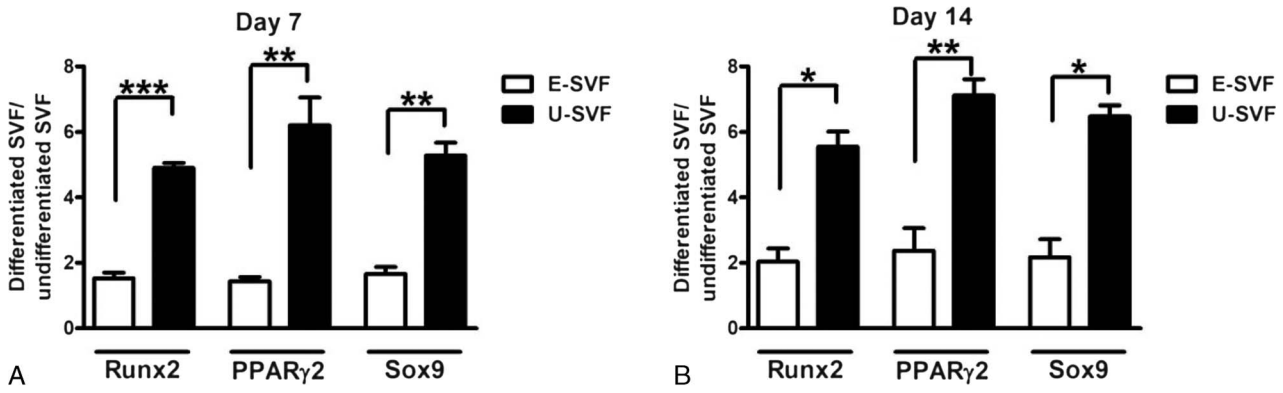

FIGURE 4. Analysis of the expression of differentiation marker genes (transcription factors Runx2, PPARγ2, and Sox9) by RT-PCR at (A) day 7 and (B) day 14. *$P < 0.05$; **$P < 0.01$. The gene expression levels of Runx2, PPARγ2, and Sox9 in differentiated SVF relative to those in undifferentiated SVF at day 7 and day 14.

analyzed by RT-PCR at 24 hours after cell seeding, is shown in Figures 6E–H. Genetic alterations of intracellular molecules FAK, RhoA, ERK, and Akt were also observed. The expression of FAK, RhoA, ERK, and Akt at 24 hours was upregulated in the fibroblast +E-SVF and fibroblast+U-SVF groups. The expression in the fibroblast+U-SVF and fibroblast+E-SVF groups was higher than that in the fibroblast group.

DISCUSSION

In this study, we first reported isolation of SVF by the ultrasonic cavitation method, and its yield, cell viability, and expression levels of stem cell characteristic proteins were comparable with those of SVF isolated by the enzymatic method. Both U-SVF and E-SVF had good differentiation ability and cell viability. Finally, SVF exosomes were cocultured with fibroblasts, and it was found that SVF exosomes strongly promoted cell migration. Generally, these data indicate that SVF can be effectively isolated by the ultrasonic method and that SVF cells and their derivatives are suitable for skin wound healing and have great therapeutic potential.

In the enzymatic method for isolating SVF, the enzymes used are obtained from pathogenic bacteria (e.g., *Clostridium difficile*); therefore, the influence of these enzymes on human health is still unknown. In addition, many countries do not permit the use of enzymes to treat human tissues or cells.[29] To overcome the shortcomings of the enzymatic method mentioned above, various methods for mechanical isolation of SVF have been studied. Research has shown that mechanical manipulation and mechanical force can enhance cell functions and effectiveness and increase the expression of cell phenotypic characteristics, thus, promoting the synthesis of specific proteins.[30] Although mechanical isolation methods can be used to obtain SVF more easily and rapidly and can also be carried out conveniently in the operating room environment, the disadvantage of mechanical isolation methods such as centrifugation, shaking, and stirring is that the cell yield is low, at only 10% of the cell amount obtained by the enzymatic method.[19] Therefore, in this study, the ultrasonic cavitation method which has been used in clinical practice was used for isolation. This method has a high isolation efficiency and requires little time, and the isolated SVF have been used in treating migraine and knee osteoarthritis.[25,31] This study confirmed that there was no significant difference in cell viability (~90%) or cell number (~85×10^4 cells/mL fat) between SVF isolated by the ultrasonic method and the enzymatic method.

Nevertheless, another study showed that the influence on cell number is not as important as previously assumed, whereas the functions and effectiveness of cells are the most important.[30] The SVF cell populations contain not only pluripotent MSCs but also hematopoietic stem cells. It is well known that ADSC markers such as CD105, CD73, and CD90 are positive, whereas CD31, CD45, CD11b, HLA-DR, CD34, and CD19 are negative, and that CD34 is mainly used to identify and distinguish hematopoietic stem cells from MSCs. Among hematopoietic cells, CD45 and CD105 are negative, but CD34, CD31, CD90, and CD106 are positive; among pericytes, CD31, CD34, CD45, and CD105 are negative, whereas CD106 and CD90 are positive.[3,32] However, the composition ratios of SVF cell populations will be affected by the different SVF isolation procedures, patient ages, downstream treatments, etc., among different laboratories.[3] In this study, approximately 65% of ADSCs and approximately 25% of ECs could be obtained by enzymatic isolation; in the ultrasonic isolation method, approximately 70% of ADSCs and approximately 20% of hematopoietic cells could be obtained at passage 0. At passage 2, the proportion of ADSCs in SVF was still higher than that in hematopoietic cells in SVF. Stromal vascular fraction consists of a heterogeneous population of stem and stromal cells, including stem cells, progenitor cells, ECs, monocyte/macrophages, smooth muscle cells, and pericytes.[2–5] The tri-differentiation ability of SVFs is mainly because of the stem cell population in SVFs.[33] The surface marker analysis in this study showed

TABLE 2. Exosome Characterization

Types	E-SVF	U-SVF	*P*
The average particle size (nm)	124.7 ± 0.1	110.4 ± 2.5	0.0013
The minimum particle size (nm)	113	76	<0.0001
No. particles (particle/mL)	$5.97 \times 10^{11} \pm 2.81 \times 10^{10}$	$9.94 \times 10^{11} \pm 5.91 \times 10^{9}$	<0.0001
CD 9 expression (%)	95 ± 3	90 ± 6	0.2663
CD 63 expression (%)	90 ± 9	97 ± 2	0.2588
CD 81 expression (%)	93 ± 5	94 ± 1	0.7512

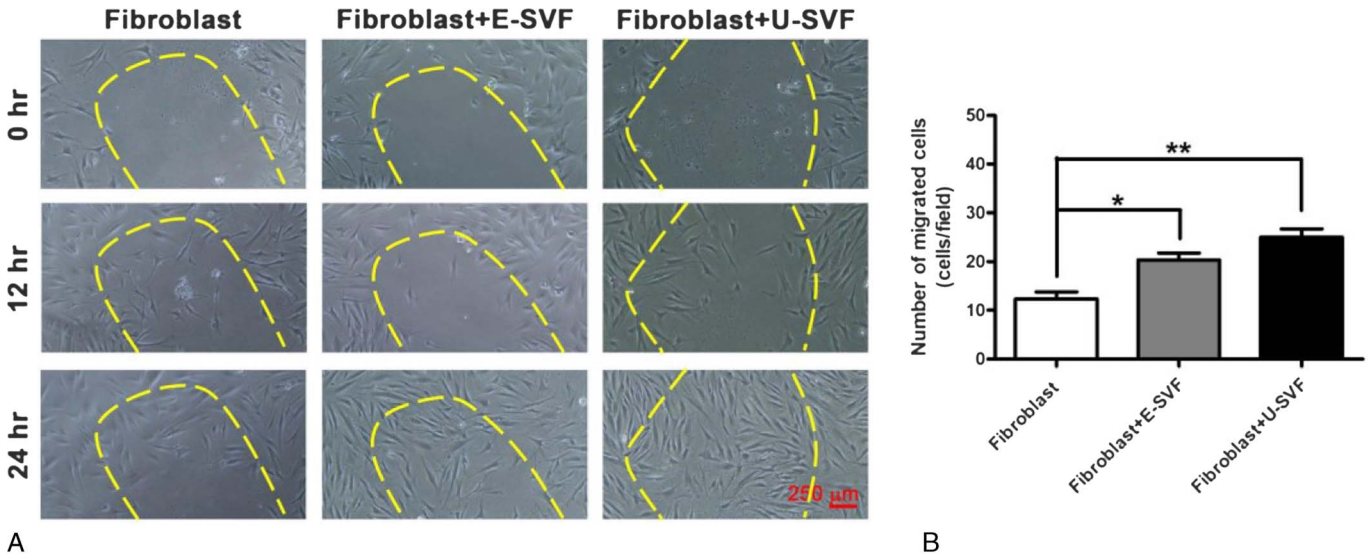

FIGURE 5. (A) Images of fibroblasts treated with E-SVF exosomes (fibroblast+E-SVF), U-SVF exosomes (fibroblast+U-SVF), and without any exosomes (fibroblast) after 0, 12, and 24 hours by wound healing assay. B, Quantitation of migrated fibroblasts after 24 hours of culture as determined by transwell assay.

that the stem cell population of U-SVF and E-SVF was greater than that of the hematopoietic cell population. Furthermore, the differentiation ability of U-SVF was greater than that of E-SVF because the number of stem cells was greater than that of the hematopoietic cell population obtained by physical methods (such as vortexing/centrifugation and dissociation by intersyringe).[33] The trilineage differentiation potential was also associated with the ADSC content in the SVF proportion. These data indicated that the isolated SVF cells contained heterogeneous cell populations. Besides surface markers, a functional criterion to define the ADSCs is their trilineage differentiation potential into the mesodermal lineages.[34] In this study, it was found that U-SVF had better differentiation potential than E-SVF after culture for 7 and 14 days. The better differentiation potential may be associated with the fact that the proportion of ADSCs was higher than that of other cell populations in U-SVF. However, U-SVF was inferior to E-SVF in terms of cell adhesion and proliferation efficiency. When

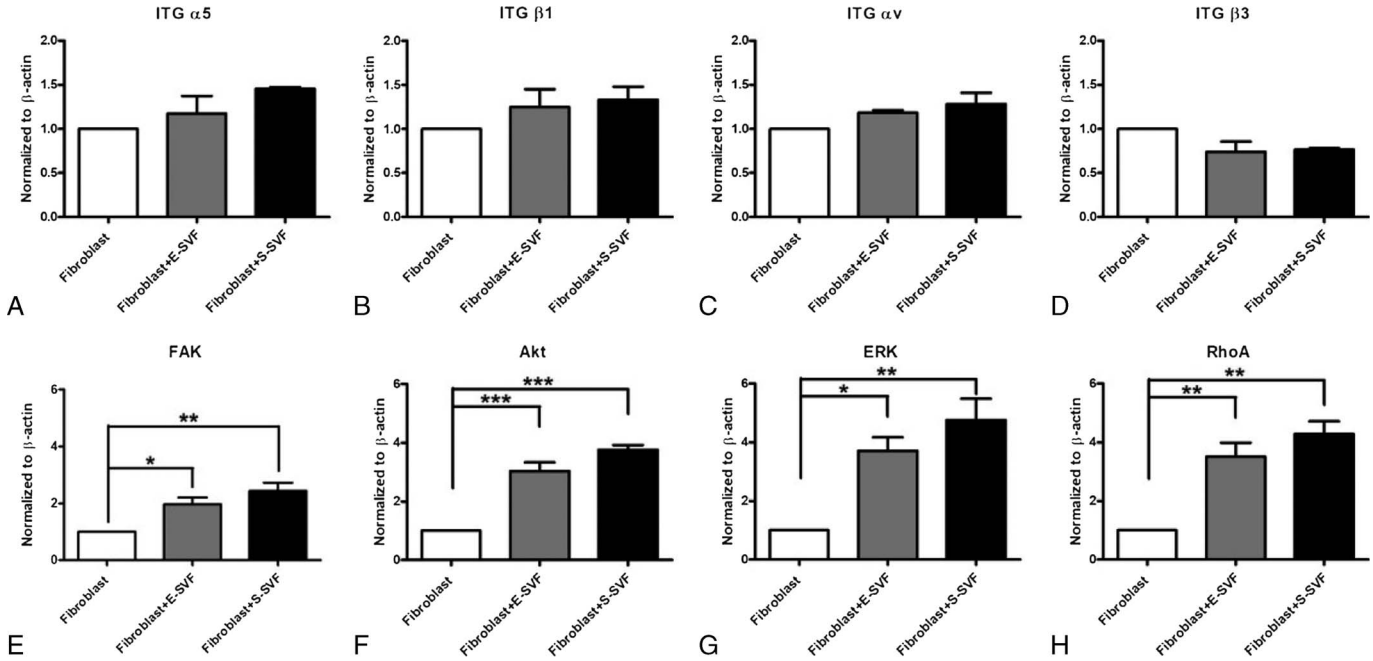

FIGURE 6. The gene expression levels of (A) integrin α5, (B) integrin β1, (C) integrin αv, (D) integrin β3, (E) FAK, (F) Akt, (G) RhoA, and (H) ERK in fibroblasts treated with E-SVF exosome, U-SVF exosome, and without exosome at 24 hours post-cell seeding. *$P < 0.05$; ***$P < 0.01$.

SVF is isolated by the manual mechanical method and enzyme digestion method, the ratio of adhered cells obtained by the enzyme digestion method was 10 times more than that obtained by the manual mechanical method.[35] Mechanical isolation was not efficient in releasing adherent cells, which was because cells adhered to the natural matrix niche.[36] However, the low adhesion rate and proliferation efficiency of U-SVF does not necessarily mean that it has low potential for therapeutic purposes. In this study, we hoped that all cell types in SVF could be isolated and collected completely by the ultrasonic isolation method, and there was no significant difference from the enzymatic method, to verify the possibility of replacing the enzymatic method with the ultrasonic method.

Wound healing is a dynamic process that includes the inflammation, proliferation, and migration of different types of cells.[37,38] From the late stage of inflammation to the complete epithelialization of injured tissues, fibroblasts play an important role in tissue repair because they can secrete extracellular matrix components such as growth factors, cytokines, and collagen.[30,39] Recent research has shown that coculture of MSCs and dermal fibroblasts in improved Boyden chambers could enhance the migration and proliferation of dermal fibroblasts.[40] Many exosomes can be extracted from the conditioned medium of cultured adipose stem cells.[41,42] Exosomes are membrane lipid vesicles, which are considered as metabolites of cells.[43] The sizes of exosomes are 30 to 150 nm,[44–46] and the surface marker proteins of exosomes were found to be CD81, CD63, and CD9.[47] However, the isolation methods used have been reported to affect the physical properties and biological content of exosomes. Exosomes isolated using ExoQuick (chemical agent) were found to be smaller than those isolated using the ultracentrifugation method, and was also found to have a different in morphology and protein content.[48] Cells preferentially uptake smaller exosomes.[48] Exosomes are known to contain mRNAs, microRNAs, and proteins,[49,50] which could promote the migration, proliferation, and collagen synthesis of fibroblasts.[41,42] The crucial growth factors and genes of skin wound healing-mediated biological processes, such as epidermal growth factor (EGF), vascular EGF, fibroblast growth factor (FGF), hepatocyte growth factor (HGF), platelet-derived growth factor, transforming growth factor beta (TGFβ1), Jagged 1, and microRNA-126, were enveloped in exosomes originating from MSCs.[51–57] Moreover, the intracellular signal transduction pathways, PI3K/Akt or FAK/ERK, are activated by growth factors and genes in various cell types and are involved in the migration of fibroblasts.[46,47,58–60] Moreover, studies have shown that exosomes derived from stem cells could help repair tissues due to their advantages of high stability, their homing ability, the easy control of dose and concentration, and the fact that they show no immune rejection.[61–64] Exosomes secreted by human ASCs could be internalized by fibroblasts, which optimize migration and proliferation, as well as the collagen synthesis and the elastic secretary capacity of fibroblasts.[41] Interestingly, cell viability and migration were significantly improved when intact exosomes were added to the cells; however, the effect was diminished when the exosome membranes were disrupted. This suggested that the exosomes have to remain intact, probably because of the exosome membrane to cell membrane interaction.[65] Thus, more studies are required in the field of molecules, such as growth factors packaged in exosomes secreted by SVF, especially with various cell culture conditions, isolation methods, and cell sources for future clinical application.

In our research, the exosome size and surface marker protein expression were consistent with those reported in the literature. We then cocultured exosomes extracted from E-SVF and U-SVF with fibroblasts and found that exosomes derived from SVF could increase the migration rate. At the same time, we also detected integrin and intracellular molecule gene expression related to cell migration. ITGβ1 could be connected with ITGα5, which affects cytoskeleton development and cell migration,[64,66] whereas ITGβ3 is related to ITGαv.[67] Among these integrins, ITGβ1α5 is the main factor affecting the migration rate of fibroblasts.[68] In this study, ITGβ1α5 gene expression was also found to increase in fibroblasts treated with SVF exosomes, and ITGα5 had a better effect on fibroblast migration rate than ITGβ1. Furthermore, we determined the intracellular gene expression of FAK, RhoA, Akt, and ERK, which are related to cell migration. We found that the expression of FAK, RhoA, Akt, and ERK genes was significantly increased in fibroblasts treated with SVF exosomes. A pervious study reported that the regulation of integrins/FAK/RhoA/Akt/ERK is time-dependent.[67] Because integrins are molecules on the cell surface, their expression may be downregulated when the protein activity is high enough. The lower expression of integrin genes at 24 hours suggested that the signals may have been transmitted into the cell and affected the expression of FAK, RhoA, Akt, and ERK genes. Therefore, we hypothesized that the SVF exosomes may contain previously mentioned biologically active proteins and genes (such as EGF, FGF, HGF, Jagged 1, and microRNA-126), which may regulate integrin expression, and then activate the intracellular molecules FAK, RhoA, Akt, and ERK. This might affect the cytoskeleton arrangement and increase the migration of fibroblasts around the wound bed, thereby shortening the time of wound healing, thus, SVF exosomes have good clinical application prospects in soft tissue wound healing.

CONCLUSION

The ultrasonic cavitation method could effectively isolate SVF and required a relatively short time. The surface characteristic proteins, cell viability, and cell yield of SVF isolated by the ultrasonic cavitation method were similar to those of SVF isolated by the enzymatic method, but it had greater differentiation potential. Furthermore, we found that exosomes secreted by SVF could accelerate the migration efficiency of fibroblasts after coculture with wound repair-related cell fibroblasts. This study shows the potential application of SVF and its derivatives in wound treatment.

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