

The Effects of Adipose Stem Cell-Conditioned Media on Fibrogenesis of Dermal Fibroblasts Stimulated by Transforming Growth Factor- β 1

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Adipose-derived stem cells (ASCs) have been shown to enhance wound healing by human dermal fibroblasts; however, the interactions between ASCs and fibroblasts during injury remain unclear. Fibroblasts were treated with ASC-conditioned medium (ASC-CM) with and without transforming growth factor- β 1 (TGF- β 1) stimulation. Fibroblast proliferation, apoptosis, differentiation and expression of extracellular matrix genes and proteins, type I collagen, and type III collagen were measured. Also, wound-healing effect of ASC-CM was verified with in vivo animal study. ASC-CM inhibited proliferation and enhanced apoptosis of fibroblasts under TGF- β 1 stimulation. Furthermore, 10% ASC-CM inhibited α -smooth muscle actin expression in fibroblasts, whereas 100% ASC-CM increased collagen, especially type III, expression in fibroblasts. ASC-CM was found to contain more basic fibroblast growth factor than hepatocyte growth factor, and 100% ASC-CM increased hepatocyte growth factor gene expression in fibroblasts. These results suggest ASCs affect fibrogenesis by dermal fibroblasts stimulated with TGF- β 1 via paracrine signaling by adipocytokines present in ASC-CM. These results also suggest that higher concentrations of ASC-CM increase collagen production and inhibit fibroblast proliferation to avoid excessive fibrogenesis. We demonstrated that a lower ASC-CM concentration attenuated fibroblast differentiation. Additionally, 100% ASC-CM significantly reduced the wound size in an in vivo wound-healing model. In this study, we provided evidence that ASCs modulate fibrogenesis by fibroblasts via paracrine signaling, suggesting that application of ASCs during wound healing may improve the quality of wound repair. (*J Burn Care Res* ;2018;39:129–140)

Debridement, which entails the removal of subcutaneous adipose tissue, after extensive burns and other kinds of trauma is performed routinely in clinical practice

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because, even though subcutaneous adipose tissue may appear healthy, it can readily develop necrosis and interfere with surgical wound healing. However, previous work in animals has demonstrated that split-skin grafts transplanted onto subcutaneous fat reduces contracture and graft tissue maturation time when compared to grafts on the muscle fascia.¹ Our previous work and an additional study have also revealed that the presence of adipose tissue alleviates fibrogenesis after injury,^{2,3} but the mechanism remains unclear.

Adipose-derived stem cells (ASCs) are pluripotent cells similar to mesenchymal stem cells derived from bone marrow that have been found to positively affect wound healing, scarring, and photoaging.^{4,5} Kim⁶ showed that secretory factors derived from ASCs promoted wound healing via human dermal fibroblasts (HDFs). In this study, we evaluated the role of ASCs on HDF proliferation, collagen synthesis, and differentiation.

Although little is known regarding the effect of ASCs on HDF fibrogenesis within a wound environment, it has been well established that the major features of fibrosis are excessive and abnormal deposition of collagen-based extracellular matrix (ECM) proteins, namely, type I collagen (Col-1) and type III collagen (Col-3), and the transformation of fibroblasts to myofibroblasts.^{7,8} Transforming growth factor- β 1 (TGF- β 1) is a known regulator of fibrinogenesis,^{9–11} and TGF- β 1-induced transcription results in accumulation of ECM proteins and overexpression of α -smooth muscle actin (α -SMA), which is involved in myofibroblast contraction, fibrosis, and scar formation. TGF- β 1 also inhibits the activities of matrix metalloproteinases, which degrade collagens and other components of the ECM. To investigate the effect of ASCs on dermal fibroblasts, dermal fibroblasts were treated with varying concentrations of ASC-conditioned medium (ASC-CM) and paracrine interactions between them were evaluated on TGF- β 1 stimulation. Our data provide evidence for the potential role of ASCs in wound repair and fibrogenesis.

METHODS

Cell Lines and Cultures

Human foreskin fibroblast (HFF-1) cells were obtained from ATCC (Manassas, Virginia, USA) (SCRC1041™) and subcultured in Dulbecco's Modified Eagle's medium (DMEM) medium (GIBCO, Paisley, United Kingdom). Human ASCs (passage 1, PT-5006) were obtained from Lonza (Walkersville, MD) and were subcultured in growth medium (Lonza). HFF-1 passages 23 to 30 and ASCs passages 2 to 5 were used in this study. To simulate the wound environment, cells were cultured in the presence of the profibrogenic cytokine TGF- β 1 as described below.

Flow Cytometric Characterization of ASCs and Differentiation of ASCs Into Adipogenic and Chondrogenic Cells

ASCs from passage 3 were incubated with Fluorescein isothiocyanate-conjugated anti-cluster of differentiation (CD)90 antibodies (1/20, Abcam, Cambridge, United Kingdom) and anti-CD105 antibodies (1/20, Abcam) or with Phycoerythrin-conjugated anti-CD34 (1/20, Abcam), anti-CD49d (1/20, Becton, Dickinson and Company), and anti-CD73 (1/20, Becton, Dickinson and Company) antibodies for 30 minutes at room temperature. As a control, cells were stained with an isotype control Immunoglobulin G (IgG). Cells were subsequently washed

with phosphate buffer saline (PBS) and analyzed on CytoFLEX S flow cytometer (Beckman Coulter, Chino, California, USA) using CytExpert software.

ASCs were then differentiated into adipogenic or chondrogenic cells using adipogenesis or chondrogenesis differentiation kits (GIBCO). The in vitro differentiation potential was determined by lineage-specific staining; adipogenic ASCs were stained with Oil Red O and chondrogenic ASCs were stained with Alcian Blue (Sigma, St. Louis, Missouri, USA).

Collagen Production in Fibroblasts After TGF- β 1 Stimulation

Fibroblasts (4×10^4 /mL) were seeded in 24-well plates with 0.5 mL per well, and the medium was replaced with serum-free DMEM/F12 (GIBCO) after cell attachment. On the following day, cells were treated with 0, 5, or 10 ng/mL TGF- β 1 (CALBIOCHEM, Darmstadt, Germany) in media to stimulate the fibroblasts. Treatment with each TGF- β 1 concentration was performed in triplicate. After 72 hours, media were discarded and 300 μ L Sirius Red was added to each well and the plates were incubated for 90 minutes. The wells were subsequently rinsed with acidified water and air-dried overnight. Sodium hydrate (0.1 M) was added to each well to resuspend the Sirius Red, the plates were shaken until the stain was completely dissolved, and absorbances were measured at 540 nm. The experiment was performed three independent times.

Preparation of ASC-CM

ASCs (4×10^5 cells) were inoculated into 75 cm² flasks containing specialized growth medium with 10% fetal bovine serum (FBS) and the flasks were incubated overnight. The following day, the medium was exchanged for serum-free DMEM/F12 and the cultures were incubated for another 72 hours. After 72-hour incubation, the conditioned medium (ASC-CM) was collected, centrifuged at 500g for 5 minutes, filtered through a 0.22 μ m syringe filter, and stored at -80°C for future use.

Treatment of HFF-1 Cells With ASC-CM

HFF-1 cells were treated with varying dilutions of ASC-CM (0, 10, 50, and 100%) in DMEM/F12 with 2% FBS (final concentration) and 5 ng/mL of TGF- β 1, and these cell cultures were incubated for 72 hours. Proliferation, apoptosis, and differentiation of HFF-1 cells treated with ASC-CM were examined as described below. Gene expression and protein abundance of fibrosis-associated factors were also evaluated as described below.

Analysis of Cytokines in ASC-CM by Enzyme-Linked Immunosorbent Assay

Concentrations of hepatocyte growth factor (HGF) and basic fibroblast growth factor (FGF-2) cytokines in ASC-CM were measured by enzyme-linked immunosorbent assay (ELISA, Abcam) according to the manufacturer's instructions. This experiment was performed two independent times.

Real-Time Cell Proliferation

Continuous monitoring of cells was performed using an xCELLigence system (ACEA, San Diego, California, U.S.A), which facilitates label-free real-time cell analysis by measuring impedance-based signals across a series of interdigitated gold electrodes. For this investigation, 50 μ L of DMEM/F12 media with 2% FBS (final concentration) and various ASC-CM dilutions were placed in the wells of the xCELLigence E-Plates and the electrodes were stabilized for 30 minutes. The plates were then moved into the xCELLigence DP analyzer to set a baseline without cells. Another 50 μ L of media containing HFF-1 cells (500 cells/well) were added to the wells of the plates. Cells were monitored for 72 hours, and impedance was read and recorded every 15 minutes through 286 sweeps. The experiment was performed three independent times.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Assay

HFF-1 cells at a density of 10^5 /mL (0.5 mL/well) in DMEM with 10% FBS were seeded into 24-well plates. After 4 hours, the cells had attached to the plates, the medium was changed to serum-free DMEM/F12 media, and the cells were incubated overnight. Subsequently, the media was replaced with various dilutions of ASC-CM, and the cells were incubated for an additional 72 hours. An APO-BRDU kit (Novus Biologicals, Littleton, Colorado, USA) was used to detect HFF-1 cell apoptosis in situ according to the manufacturer's instructions. The experiment was performed four independent times.

Real-Time Polymerase Chain Reaction Analysis

RNA was isolated from HFF-1 cells on incubation in ASC-CM for 4 hours with Trizol reagent (Invitrogen, Carlsbad, California, U.S.A) using standard methods. Complementary DNA (cDNA) was synthesized by reverse transcription using a cDNA synthesis kit (Invitrogen). Real-time quantitative polymerase chain reactions (PCRs) were performed in triplicate using

Table 1. Primers for real-time PCR analysis

Gene		Primer Sequence 5'→3'
GAPDH	F	TCTTTTGCCTCGCCAGCCGAG
	R	TGACCAGGCGCCCAATACGAC
Col-1 α 1	F	ACGAAGACATCCCACCAATC
	R	AGATCACGTCATCGACAAC
Col-3 α 1	F	GCCTCCCGGAAGTCAAGGAGAAA
	R	CTTTAGGACCGGGGAAGCCCATG
α -SMA	F	CTGCTGAGCGTGAGATTGTC
	R	CTCAAGGGAGGATGAGGATG
HGF	F	ATGTCAGCCCTGGAGTTCCATGAT
	R	AGCGTACCTCTGGATTGCTTGTGA
FGF-2	F	CTGGCTATGAAGGAAGATGGA
	R	TGCCAGTTCGTTTCAGTG

α -SMA, α -smooth muscle actin; Col-1 α 1, collagen type 1 α 1; Col-3 α 1, collagen type 3 α 1; FGF-2, basic fibroblast growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HGF, hepatocyte growth factor.

SYBR Green Real-time PCR Master Mix (Applied Biosystems) and run on an Applied Biosystems 7500 Real-time PCR machine. The PCR primers were designed based on cDNA sequences in the National Center of Biotechnology Information (NCBI) database (Table 1). Gene expression data for Col-1 α 1, Col-3 α 1, α -SMA, HGF, and FGF-2 were normalized to the internal control gene, glyceraldehyde 3-phosphate dehydrogenase. Data were analyzed with the $2^{-\Delta\Delta C_t}$ method, and the experiment was performed four or five independent times.

Western Blot Analysis

Regarding the abundance of collagens I and III expression, aliquots of culture media were collected and 5 μ g of protein were extracted from fibroblasts to evaluate α -SMA expression. Proteins were separated using NuPAGE[®] 4 to 12% Bis-Tris gels (Novex, Paisley, United Kingdom) and transferred onto nitrocellulose membranes. After blocking with Odyssey blocking buffer (LI-COR Biosciences, Cambridge, United Kingdom), the membranes were incubated with antibodies against human collagen type I or III (1/1000, Abcam), α -SMA (1/200, Abcam), and β -actin (1/1000, Abcam) at 4°C overnight. Then, membranes were washed with tris buffered saline with Tween (TBS-T) and incubated with an Alexa Fluor-conjugated secondary antibody (1/1000, Abcam) at 4°C for 1 hour. Immunoreactive bands were visualized with an Odyssey Infrared Scanner (LI-COR Biosciences). The intensity of each band was determined using ImageJ software (version 1.48v; National Institutes of Health, Bethesda, MD). The experiment was performed three independent times.

In Vivo Animal Wound-Healing Model

Seven male Sprague Dawley rats weighting 200 to 250 g were purchased from Shanghai Laboratory Animal Center in the Chinese Academy of Sciences and housed under specific pathogen-free conditions in animal facility. This study was performed in strict accordance with the standards established by

the guidelines for the Care and Use of Laboratory Animals of Shanghai Jiao Tong University and was approved by the laboratory Animal Ethics Committee of Ruijin Hospital (Permit Number: 190). Rats were anaesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg body weight). The backs of the rats were shaved, and the

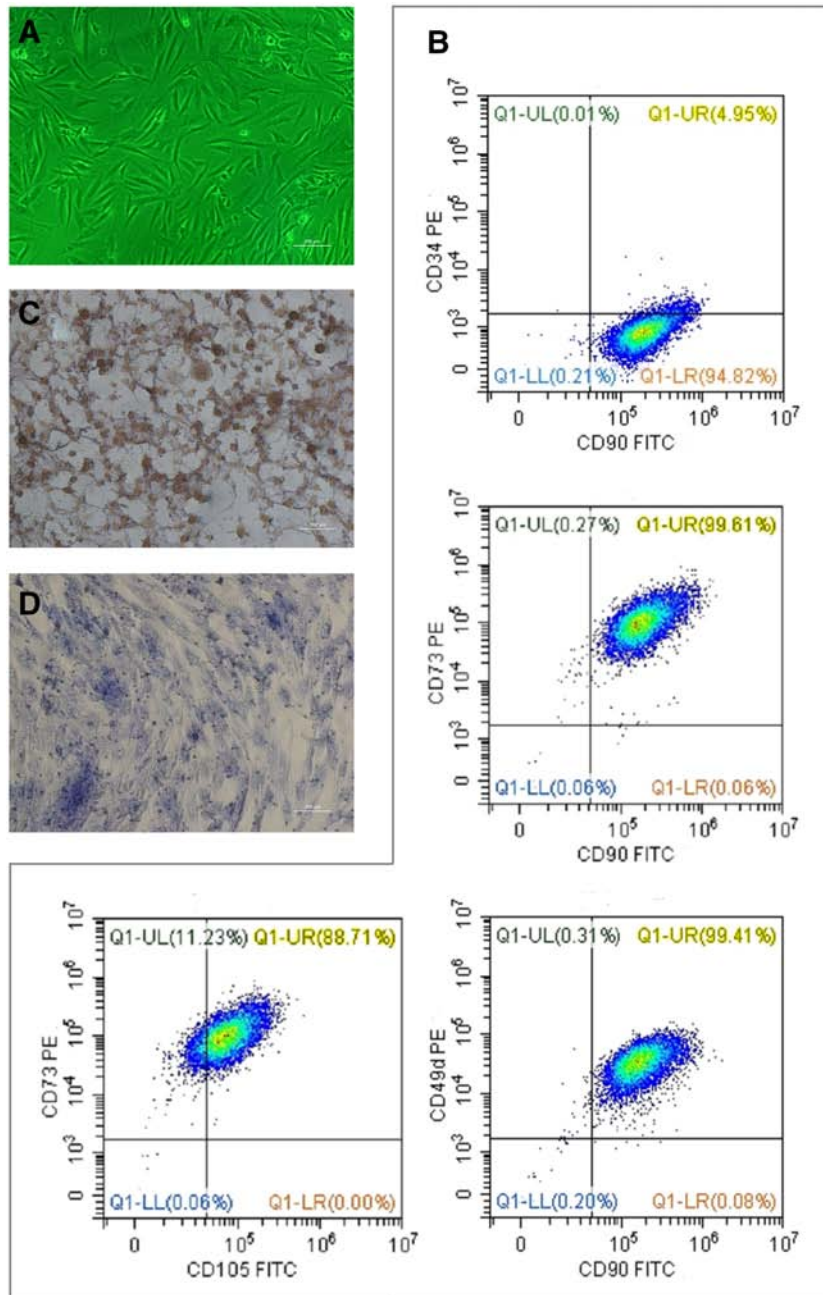


Figure 1. Characterization of cell surface markers of ASCs by flow cytometry and differentiation of ASCs. Microscopy demonstrated that the ASCs have a fibroblast-like morphology (A). Flow cytometry showed that ASCs were positive for CD49d, CD73, CD90, and CD105 but were negative for CD34. (B). Fourteen days after induction, ASCs rounded and lipids collected in cytoplasm, which was confirmed by positive-staining with Oil Red O (C); in chondrogenesis differentiation medium, ASCs formed chondrogenic pellets, which were stained with Alcian Blue (D). Scale bars: 100 μ m. ASC, adipose-derived stem cells.

hairs were thoroughly removed by (Nair; Church & Dwight, Australia). Using a 6-mm punch biopsy instrument (Acuderm Inc., Fort Lauderdale, FL), six full-thickness skin wounds, paralleling to the spine, were symmetrically created on the back of each animal. Wounds were left uncovered following surgery. Rats were anesthetized with sodium pentobarbital on days 3 to 10 after the wounding. Meanwhile, four wounds were applied with 0, 10, 50, and 100% ASC-CM individually and the remaining two were allowed to heal spontaneously as blank controls. At each time point, the wounds were photographed and drawn on the transparent tracing paper. The paper was then scanned and the wound size was analyzed using ImageJ software. The unhealed rate was calculated by comparing the unhealed wound area to the original wound area. Eventually, the unhealed rate of each group was normalized by the blank control in the same animal.

Statistical Analysis

All data are presented as the mean \pm standard deviation. Groups were compared using one-way analysis of variance with Tukey's post hoc test. Linear relationships were assessed using the Pearson correlation. A P value of $<.05$ was considered statistically significant.

RESULTS

Characterization of ASCs

ASCs exhibited fibroblast-like morphology (Figure 1A), and characteristic expression of stem cell-related surface markers was confirmed by flow cytometry. ASCs expressed CD49d, CD73, CD90, and CD105 markers but lacked the CD34 marker (Figure 1B).

Fourteen days after adipogenesis was induced, most of the ASCs stained positive for Oil Red O, suggesting that they contained lipids, which is an indicator of adipocytes (Figure 1C). Differentiation of ASCs into chondrocytes was verified by Alcian Blue staining (Figure 1D). Staining of nondifferentiated ASCs with these differentiation markers was negative (data not shown).

Collagen Production in Fibroblasts Increased After Stimulation With 5 or 10 ng/mL of TGF- β 1

Stimulation with 5 and 10 ng/mL of TGF- β 1 for 72 hours led to an increase in collagen production in fibroblasts when compared to the control. Furthermore, stimulation with 10 ng/mL of TGF- β 1 led to the production of more collagen than

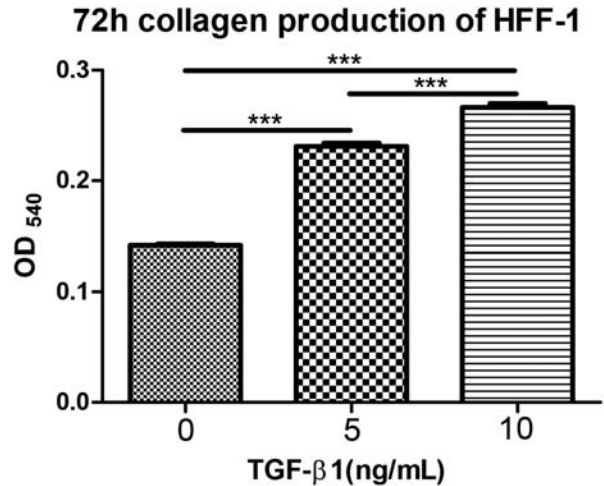


Figure 2. Quantification of collagen production by HFF-1 cells after 72 hours of stimulation with TGF- β 1 was performed by Sirius Red staining. Collagen production was increased with 5 and 10 ng/mL of TGF- β 1 when compared with the control group (0 ng/mL of TGF- β 1). More collagen was produced with 10 ng/mL of TGF- β 1 than with 5 ng/mL of TGF- β 1. Solid bars represent analysis of variance with Tukey's posttest among groups. * P value $<.001$; $n = 3$. HFF-1, human foreskin fibroblast; TGF- β 1, transforming growth factor- β 1.

stimulation with 5 ng/mL of TGF- β 1, suggesting that TGF- β 1 enhancement of collagen production in fibroblasts is concentration dependent (Figure 2). *ASCs inhibit fibroblast proliferation and enhance fibroblast apoptosis via paracrine on TGF- β 1 stimulation.*

HFF-1 cell proliferation was enhanced with 0 to 44 hours of TGF- β 1 treatment, but after 72 hours of TGF- β 1 stimulation, proliferation was attenuated. In addition, it was observed that, under TGF- β 1 stimulation, HFF-1 cell proliferation was inhibited by ASC-CM in a dose-dependent manner (Figure 3). Furthermore, in the absence of TGF- β 1 stimulation, the inhibitory effect of ASC-CM (100%) on HFF-1 proliferation was diminished demonstrating that TGF- β 1 and ASC-CM attenuate cell proliferation synergistically (Figure 3). To further understand this response, apoptosis was quantified by transferase deoxyuridine triphosphate (dUTP) nick end labeling analysis, and apoptosis of HFF-1 cells under TGF- β 1 stimulation increased as ASC-CM concentrations increased (Pearson correlation $r^2 = .9751$, $P = .0125$; Figure 4). HFF-1 cells with 100% ASC-CM and TGF- β 1 stimulation had the highest level of apoptosis, whereas HFF-1 cells without TGF- β 1 stimulation tended to have lower apoptotic signals, although this difference was not significant (Figure 4).

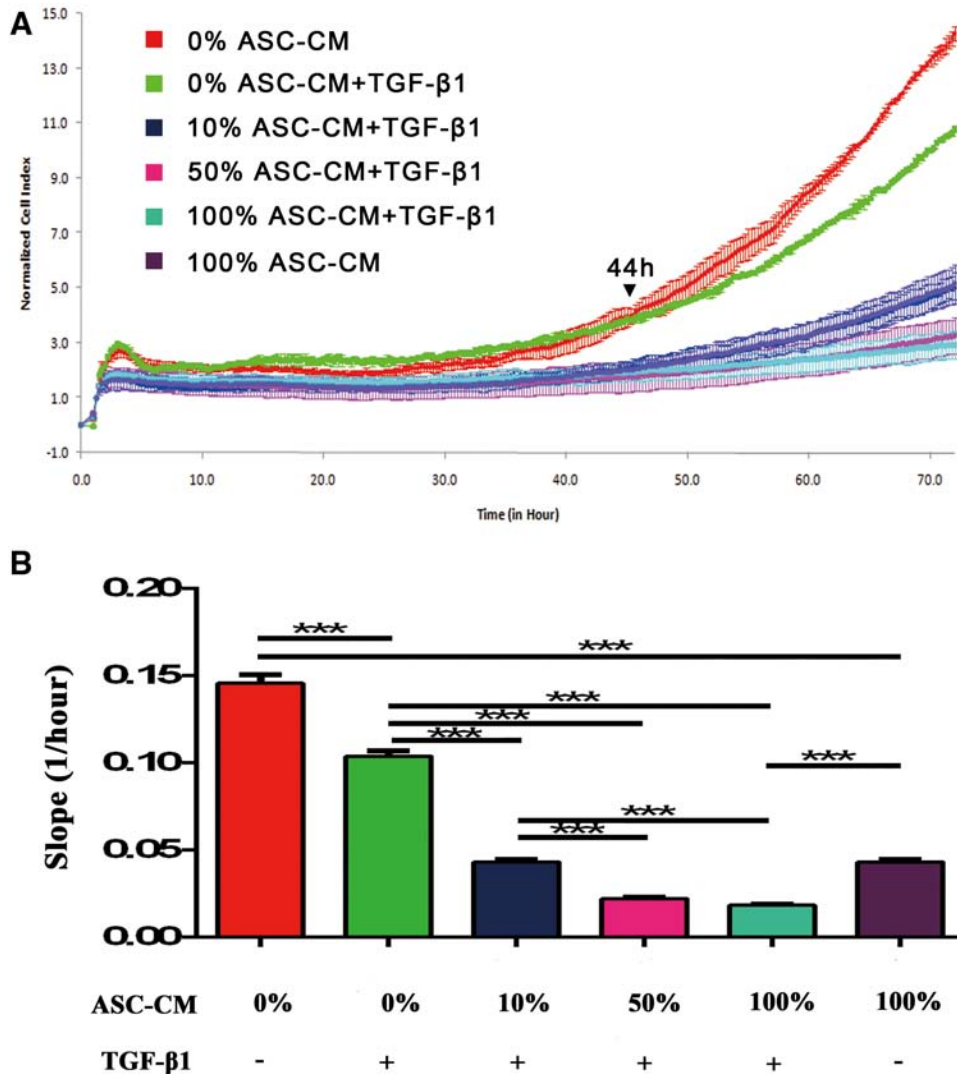


Figure 3. Real time of HFF-1 cell proliferation in ASC-CM was monitored using an xCELLigence (ACEA) platform. All of the experiments were performed using E-plates according to the protocol in the ACEA manual and N = 3. (A and B) HFF-1 cells were seeded at 500 cells/well with or without ASC-CM and TGF-β1 (with appropriate controls and blanks). Solid bars represent analysis of variance with Tukey’s posttest among groups. *P value < .001; n = 3. ASC-CM, adipose-derived stem cells–conditioned medium; HFF-1, human foreskin fibroblast; TGF-β1, transforming growth factor-β1.

10% ASC-CM May Decrease α-SMA Expression in Fibroblasts

TGF-β1 treatment increased α-SMA gene expression without ASC-CM (Figure 5). With TGF-β1 stimulation and 10% ASC-CM, α-SMA gene expression was significantly lower than with TGF-β1 and 0% ASC-CM. α-SMA protein abundance data among the different groups were concordant with α-SMA gene expression data. α-SMA expression decreased in the group treated with 10% ASC-CM when compared to the groups treated with 0% ASC-CM and 50% ASC-CM with TGF-β1 stimulation indicating that 10% ASC-CM attenuates TGF-β1–induced cell differentiation (Figure 5).

100% ASC-CM Promotes Collagen Expression and Secretion by Fibroblasts

Col-1 α1 and Col-3 α1 gene expression exhibited similar trends (Pearson correlation $r^2 = .853$, $P = .031$). Specifically, TGF-β1 increased Col-1 α1 and Col-3 α1 gene expression when comparing the 0% ASC-CM group with and without TGF-β1 and when comparing the 100% ASC-CM group with and without TGF-β1. Among the groups with TGF-β1 stimulation, Col-1 α1 gene expression in the groups treated with 10% and 50% ASC-CM was lower than in the groups treated with 0% and 100% ASC-CM. Col-3 α1 gene expression was lower with 10% and 50% ASC-CM than with 100% ASC-CM. Collagen

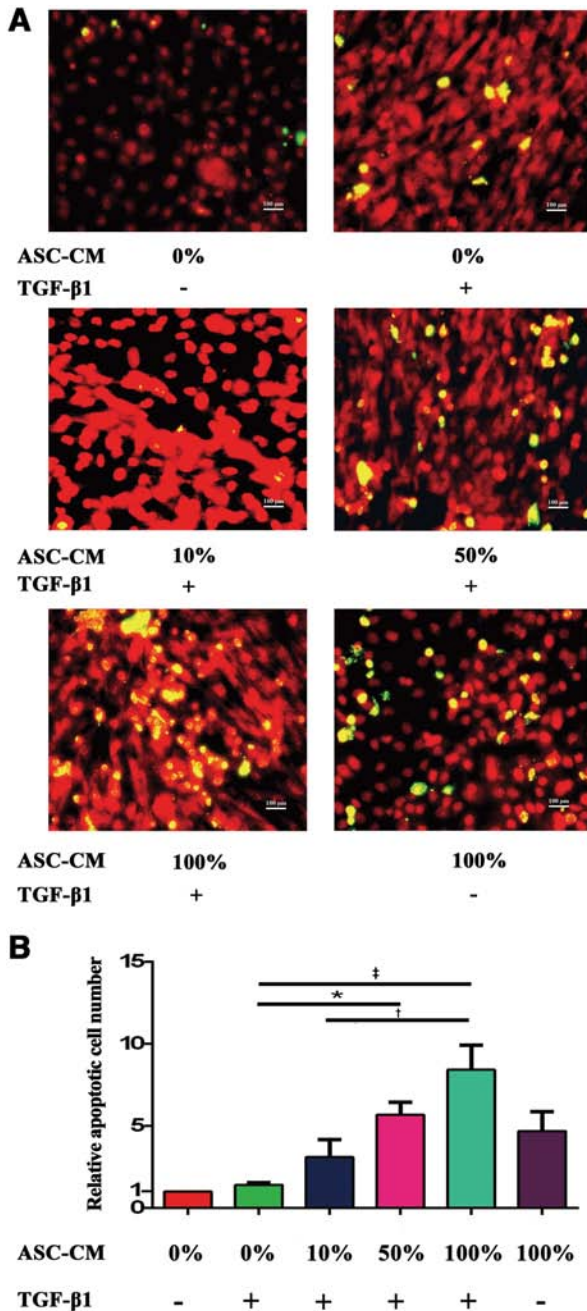


Figure 4. Analysis of HFF-1 cell apoptosis in ASC-CM. (A) Visualization of apoptotic HFF-1 cells with various dilutions of ASC-CM. The red stain (propidium iodide) indicates the nucleus. The green (fluorescein-labeled anti-dUTP antibody) and the yellow (derived by merging green and red) stains indicate TUNEL-positive HFF-1. (B) Quantitative data from the 72-hour endpoint demonstrated that increasing ASC-CM concentrations promoted apoptosis. Solid bars represent analysis of variance with Tukey's post-test among groups. **P* value < .05; †*P* value < .01; ‡*P* value < .001; *n* = 4. Scale bars: 100 μm. ASC-CM, adipose-derived stem cells-conditioned medium; HFF-1, human foreskin fibroblast; TUNEL, transferase dUTP nick end labeling.

protein was also quantified in the supernatants of all of the groups, and Col-3 production was higher than Col-1 production. Moreover, Col-3 production in supernatants was highest in the group treated with 100% ASC-CM when compared with the groups treated with 10% and 50% ASC-CM (Figure 6).

ASC-CM Contained More FGF-2 Compared With HGF, and 100% ASC-CM Promotes HGF Gene Expression

ASCs produce a variety of growth factors, and we quantified the amounts of FGF-2 and HGF growth factors in ASC-CM by ELISA. We found 616.55 ± 19.25 pg/mL of FGF-2 and 33.24 ± 1.24 pg/mL of HGF in ASC-CM; thus, there was approximately 18 times as much FGF-2 than HGF in ASC-CM (Table 2).

With TGF-β1 stimulation, FGF-2 gene expression increased with 10, 50, and 100% ASC-CM when compared to 0% ASC-CM. However, there was no difference among the groups treated with 10, 50, and 100% ASC-CM. These data indicate that the combination of TGF-β1 and ASC-CM promotes FGF-2 gene expression. It should be noted that FGF-2 expression was lower with 100% ASC-CM alone than with 100% ASC-CM and TGF-β1 stimulation, suggesting that TGF-β1 may be a critical regulator of FGF-2 expression in fibroblasts. With TGF-β1 stimulation, HGF gene expression increased as ASC-CM concentrations increased (Pearson correlation $r^2 = .9439$, $P = .0284$). HGF expression was higher with 100% ASC-CM than in the media only control, demonstrating that 100% ASC-CM induced HGF expression (Figure 7).

100% ASC-CM Enhances Wound Healing In Vivo

To confirm the practical role of ASC-CM within the in vivo wound environment, the wounds were treated with different concentrations of ASC-CM including 0, 10, 50, and 100% and the untreated wound was considered as internal control. Compared with the blank control and 0% ASC-CM group, 100% ASC-CM treatment significantly accelerated wound healing on the seventh day after wounding (Figure 8). Wound closure was almost achieved within 14 days.

DISCUSSION

The commercially acquired ASCs used in the study were tested to ensure that they were typical ASCs. We demonstrated, by flow cytometry, that our ASCs were positive for CD49d, CD73, CD90, and CD105

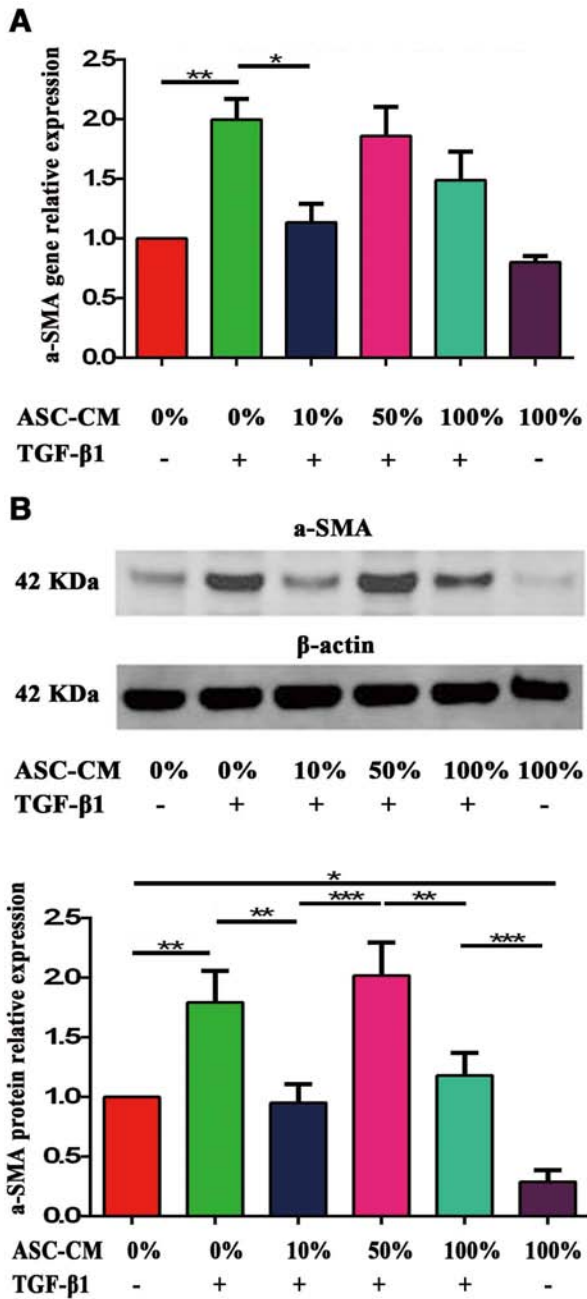


Figure 5. Expression of α -SMA in fibroblasts in ASC-CM. α -SMA gene (A) or protein (B) expression was measured by real-time PCR or Western blot with TGF- β 1 stimulation with varying ASC-CM concentrations and with of ASC-CM alone. α -SMA expression was inhibited with TGF- β 1 stimulation and 10% ASC-CM. Solid bars represent analysis of variance with Tukey's posttest among groups. * P value < .05; † P value < .01; ‡ P value < .001; $n = 5$ (A) and $n = 3$ (B). α -SMA, α -smooth muscle actin; ASC-CM, adipose-derived stem cells–conditioned medium; PCR, polymerase chain reaction; TGF- β 1, transforming growth factor- β 1.

but were negative for CD34. It has been reported that human ASCs exhibit the typical results for cell surface markers as mesenchymal stem cells, including positive results for CD90 and highly negative results for CD34.¹² In addition, 100% of the undifferentiated adipose stem cells expressed CD73 and CD105.¹³ In this study, more than 88% of the cells were CD73+/CD105+. Furthermore, our ASCs could differentiate into adipocytes and chondrocytes in specific media, suggesting that the ASCs used in this study are representative of typical ASCs.

ASCs are tissue-specific progenitor cells that have been shown to play a role in wound healing.¹⁴ These progenitor cells were shown to significantly accelerate tissue repair, reduce fibrosis, and eventually improve organ function in a mouse injury model.³ Furthermore, the mechanisms of ASC function in wound healing was demonstrated to involve growth factors secreted by ASCs via the paracrine pathway resulting in enhanced collagen production.⁶ Previous studies showed that TGF- β 1 in ASC-CM accelerated collagen deposition¹⁵ and that TGF- β 1–treated ASC-CM promoted expression of type I collagen.¹⁶ However, ASCs implanted with poly(3-hydroxybutyrate-co-hydroxyvalerate) structures reduced TGF- β 1 expression, but increased TGF- β 3 expression, which is responsible for inhibiting scarring, at the wound site.^{17,18} Although these results provide valuable insights regarding the role of ASCs in wound healing, much is yet to be determined regarding the role and mechanism of ASCs on fibrogenesis in order to understand the role of ASCs in vivo.

A critical point to consider in this experimental design is that fibroblasts are not quiescent and do not exist in isolation in vivo. Indeed, fibroblasts are likely substantially modified by the surrounding microenvironment after injury. Among the many cytokines and growth factors involved in wound healing that could affect local fibroblasts, TGF- β 1 has the broadest spectrum of effects.¹⁹ Presumably, platelets are the primary source of activated TGF- β 1, which accumulates at the site of tissue damage immediately after injury.²⁰ In addition, TGF- β 1 is the predominant growth factor in adults and has been shown to be responsible for the fibrotic scarring response.^{21,22} Therefore, 5 ng/mL TGF- β 1 was introduced into our cell culture system to simulate a wounded tissue environment. Cellular events, such as proliferation, apoptosis, differentiation, and collagen production, were evaluated to determine the role of ASCs in promoting or alleviating fibrogenesis. In addition, the effect of ASC-CM on wound healing was also evaluated in an animal injury model.

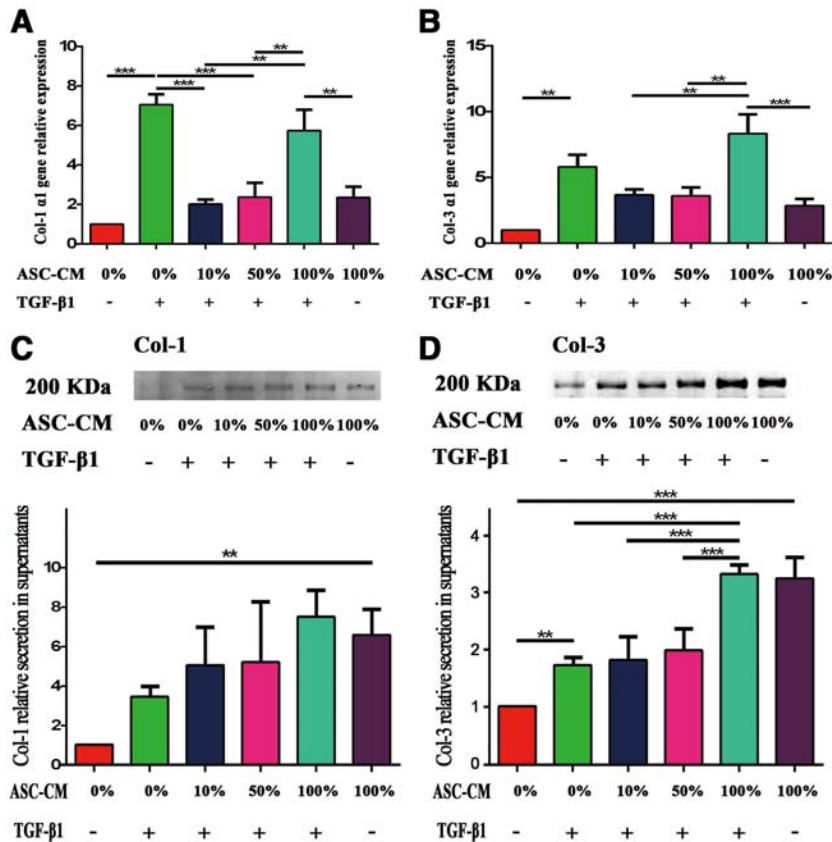


Figure 6. Expression of collagen types 1 and 3 by fibroblasts in ASC-CM. (A) Col-1 $\alpha 1$, (B) Col-3 $\alpha 1$ gene expression in HFF-1 cells and (C) Col-1, (D) Col-3 protein secretion in supernatants were measured by real-time PCR or Western blot with TGF- $\beta 1$ stimulation with varying ASC-CM concentrations and with ASC-CM alone. Col-3 gene expression and protein secretion increased with 100% ASC-CM and TGF- $\beta 1$. Solid bars represent analysis of variance with Tukey's posttest among groups. * P value < .05; † P value < .01; ‡ P value < .001; $n = 5$ (A, B) and $n = 3$ (C, D). ASC-CM, adipose-derived stem cells-conditioned medium; Col-1 $\alpha 1$, collagen type 1 $\alpha 1$; Col-3 $\alpha 1$, collagen type 3 $\alpha 1$; HFF-1, human foreskin fibroblast; PCR, polymerase chain reaction; TGF- $\beta 1$, transforming growth factor- $\beta 1$.

The results of real-time cell quantification showed that fibroblast proliferation decreased as the ASC-CM concentration increased. Furthermore, the results of the transferase dUTP nick end labeling assay showed that the rate of apoptosis of fibroblasts also increased as the ASC-CM concentration increased. Although the mechanism of apoptosis was not examined in this study, further investigation showed that HGF gene expression in cells with TGF- $\beta 1$ stimulation increased as ASC-CM concentrations increased (Pearson correlation $r^2 = .9439$, $P = .0284$).

Table 2. FGF-2 and HGF in ASC-CM

Cytokines	Concentration (pg/mL)
FGF-2	616.55 \pm 19.25
HGF	33.24 \pm 1.24

ASC-CM, adipose-derived stem cells-conditioned medium; FGF-2, basic fibroblast growth factor; HGF, hepatocyte growth factor.

HGF has been shown to attenuate renal fibrosis by inducing apoptosis of myofibroblasts,²³ suggesting that HGF may play a role in mediating the ASC-CM proapoptotic effect on fibroblasts. In addition, it is known that FGF-2 is released during the early stage of wound healing, whereas HGF is expressed during the later stage,²⁴ and FGF-2 promotes HGF expression.³ In this study, gene expression analysis showed that TGF- $\beta 1$ promoted FGF-2 gene expression, which further enhanced HGF gene expression. Thus, these cytokines work as a network balancing their specific biological influences to achieve a net result,²⁵ and the network involving TGF- $\beta 1$, FGF-2, and HGF may regulate apoptosis of fibroblasts. Furthermore, ELISA quantification of FGF-2 and HGF showed that the ASC-CM contained more FGF-2 than HGF. Therefore, it is reasonable, in the future, to explore whether these two cytokines play dominant roles in fibroblast apoptosis induced

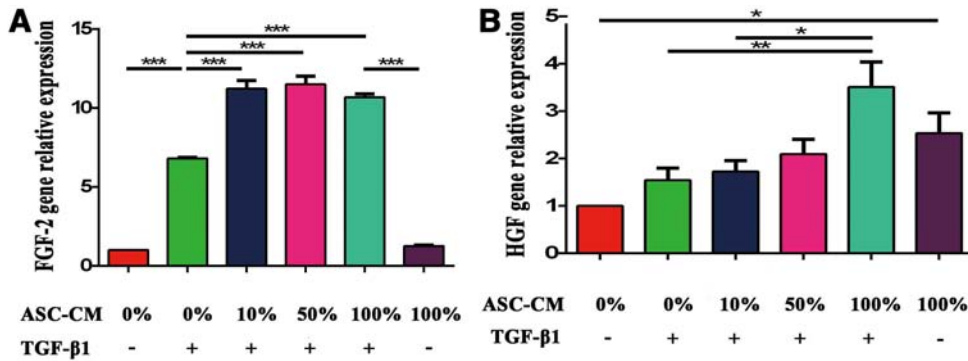


Figure 7. FGF-2 and HGF gene expression in fibroblasts in ASC-CM. (A) FGF-2, (B) HGF were measured by real-time PCR. When comparing groups treated with and without TGF-β1, TGF-β1 appears to be a crucial regulator FGF-2 gene expression. There was a close relationship between concentrations of ASC-CM and HGF gene expression. 100% ASC-CM increases HGF gene expression relative to 0 and 10% ASC-CM. Solid bars represent analysis of variance with Tukey’s posttest. **P* value < .05; †*P* value < .01; ‡*P* value < .001; *n* = 4 (A) and *n* = 5 (B). ASC-CM, adipose-derived stem cells–conditioned medium; FGF-2, basic fibroblast growth factor; HFF-1, human foreskin fibroblast; HGF, hepatocyte growth factor; PCR, polymerase chain reaction; TGF-β1, transforming growth factor-β1.

by ASC-CM. α-SMA was used as a marker of fibroblast differentiated into a myofibroblast. Compared with fibroblasts, myofibroblasts produce more collagen and promote fibrogenesis, thus they play an important role in wound healing.²⁶ In this study, we found that 100% ASC-CM inhibited α-SMA expression in the absence of TGF-β1, suggesting that 100% ASC-CM itself has the ability to inhibit fibroblast differentiation. In addition, 10%ASC-CM and TGF-β1 decreased α-SMA expression when compared with 0% ASC-CM and TGF-β1, demonstrating that a lower ASC-CM concentration, such as 10% vol/vol, rather than higher 50 and 100% ASC-CM

concentrations, inhibits fibroblast differentiation under TGF-β1 stimulation. This may explain why the 10% ASC-CM group produced the least shown Col-3, although the mechanism is unclear. Collagen types 1 and 3 are major components of ECM, and Col-3 is the primary constituent of early stage ECM, whereas Col-1 is a vital component in late stage ECM.^{27,28} Western blot analysis of cells after 72 hours of incubation showed higher amounts of Col-3 than Col-1, indicating that the 72-hour time point represents an early stage of wound healing. In addition, Col-1 α1 and Col-3 α1 expression increased with TGF-β1 and 100% ASC-CM. Moreover, the amounts of Col-3

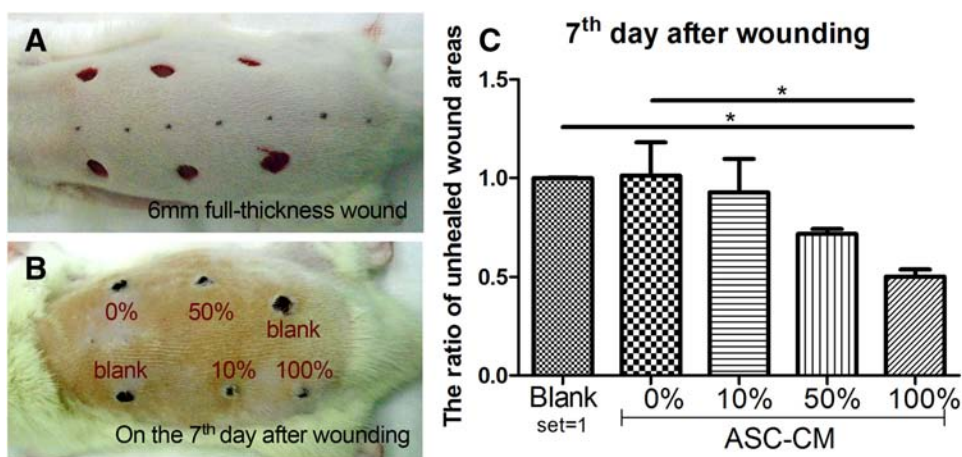


Figure 8. 100% ASC-CM promoted wound healing in an animal injury model. (A) Six 6-mm full-thickness wounds were created on the back of Sprague Dawley rats. Four of them were treated daily with 0, 10, 50, and 100% ASC-CM, respectively, since the third day after wounding, and the remaining two were allowed to heal naturally. (B) On the seventh day after wounding, the wound size seemed to be correlated with different treatments. (C) Quantitative data from the 7 days after wounding demonstrated that 100% ASC-CM significantly enhanced wound healing, compared with blank control or 0% ASC-CM group. Solid bars represent analysis of variance with Tukey’s posttest. **P* value < .05; *n* = 7. ASC-CM, adipose-derived stem cells–conditioned medium.

in the supernatants increased with 100% ASC-CM and TGF- β 1. The data of wound healing in vivo showed application of 100% ASC-CM significantly promoted wound closure, which was in accord with the results in vitro, demonstrating that 100% ASC-CM plays a positive role in wound healing within a wound environment.

Overall, 10% ASC-CM alleviated fibrogenesis by inhibiting fibroblast differentiation, but 100% ASC-CM may have dual roles in regulating fibrogenesis. It has been shown that increased survival of myofibroblasts leads to greater scar formation,²⁹ and we found that 100% ASC-CM accelerated apoptosis of fibroblasts, which may effectively reduce the numbers of fibroblasts in the wound bed, thereby preventing excessive tissue deposition. In addition, 100% ASC-CM also promoted collagen production, which may promote not only tissue repair during the early stage of wound healing but also scar formation during the late stage of wound healing. This paradoxical phenomenon indicates regulation of early- and late-phase wound-healing events; perhaps 100% ASC-CM facilitated collagen production to meet the requirements of early wound healing and simultaneously induced fibroblast apoptosis as a self-regulatory mechanism, which could optimize the degree of fibrogenesis. Furthermore, 100% ASC-CM promoted HGF gene expression, and HGF has been shown to function as an antifibrotic factor during pulmonary, renal, and liver fibrosis^{30–32} and has been shown to inhibit cardiac fibroblast proliferation and transformation in response to TGF- β 1 stimulation.³³ These data suggest that HGF plays a substantial role in regulation of fibrogenesis; thus, the role of HGF in the biology of ASCs and fibrogenesis warrants further investigation.

Some questions remain unresolved in this study. First, the discrepancy between Col-1 (or Col-3) gene and protein expression in 0% and 100% ASC-CM with TGF- β 1 is difficult to explain. However, because ASC-CM induces apoptosis of fibroblasts in a dose-dependent manner, we speculate that cell vitality influenced the ability of the cell to express collagen; further study is required to support this hypothesis. Additionally, Figure 2 showed that TGF- β 1 increased collagen levels, but Figure 3 demonstrated fibroblast proliferation decreased on addition of TGF- β 1. This begged the question: Is TGF- β 1-induced collagen production independent of cell number? To answer this question, fibroblast proliferation was monitored using an xCELLigence system and cellular morphology was analyzed by microscopy. In the xCELLigence system, fibroblast proliferation was enhanced after 0 to 44 hours of TGF- β 1

treatment, but 72 hours of TGF- β 1 stimulation attenuated proliferation. Interestingly, by microscopy, we detected numerous “cycle-like” structures between 44 and 72 hours after stimulation (data not shown), which may lead to further morphological changes, such as more stretching. Thus, TGF- β 1 may play dual roles in regulating HFF-1 proliferation. Initially, TGF- β 1 may stimulate proliferation of HFF-1 cells, and subsequently, when cells become more and more crowded, especially during cell stretching, TGF- β 1 may attenuate HFF-1 proliferation. Nevertheless, Sirius Red staining and Western blot analysis showed that TGF- β 1 increased collagen production. Currently, ASCs are easily acquired from liposuction aspirates, and they may be able to reasonably control the degree of fibrogenesis. This control is likely mediated by the cytokines produced by the ASCs. Application of ASCs or their derivatives on wounds may promote quality by inhibiting hypertrophic scar formation; thus, ASCs may function as a promising therapeutic.

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