



Wound healing effect of adipose-derived stem cells: A critical role of secretory factors on human dermal fibroblasts

Won-Serk Kim^{a,b}, Byung-Soon Park^{a,c,*}, Jong-Hyuk Sung^a, Jun-Mo Yang^d,
Seok-Beom Park^c, Sahng-June Kwak^e, Jeong-Soo Park^{a,e,*}

^a Division of Stem Cell Research, Prostemics Research Institute, Seoul, Republic of Korea

^b Department of Dermatology, Kangbuk Samsung Hospital, School of Medicine, Sungkyunkwan University, Seoul, Republic of Korea

^c Leaders Clinic, Seoul, Republic of Korea

^d Department of Dermatology, Samsung Medical Center, School of Medicine, Sungkyunkwan University, Seoul, Republic of Korea

^e Department of Biochemistry, College of Medicine, Dankook University, Chunan, Republic of Korea

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KEYWORDS

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Summary

Background: Adipose-derived stem cells (ADSCs) are a population of pluripotent cells, which have characteristics similar to bone marrow-derived mesenchymal stem cells. Whereas ADSCs have potential applications for the repair and regeneration of various damaged tissues, few studies have dealt with the effect of ADSCs on fibroblasts, which play a key role in skin biology.

Objective: In this study, we investigated the possible roles of ADSCs in skin wound healing process, especially in the aspect of fibroblast activation—proliferation, collagen synthesis and migratory properties.

Methods and results: ADSCs promoted human dermal fibroblast (HDF) proliferation, not only by cell-to-cell direct contact, which was confirmed by co-culture experiment, but also by paracrine activation through secretory factors, resolved by trans-well co-culture and culturing with conditioned medium of ADSCs (ADSC-CM). ADSC-CM enhanced the secretion of type I collagen in HDFs by regulating the mRNA levels of extracellular matrix (ECM) proteins: up-regulation of collagen type I, III and fibro-

* Corresponding authors at: Division of Stem Cell Research, Prostemics Research Institute, 60-6, Nonhyun-dong, Kangnam-gu, Seoul 135-010, Republic of Korea. Tel.: +82 2 545 2818; fax: +82 2 545 2819.

E-mail addresses: prostem@empal.com (B.-S. Park), prostem@empal.com (J.-S. Park).

nectin and down-regulation of MMP-1. Moreover, ADSC-CM showed stimulatory effect on migration of HDFs in *in vitro* wound healing models. Additional to those *in vitro* evidences, wound healing effect of ADSCs was also verified with *in vivo* animal study, resulted that ADSCs significantly reduced the wound size and accelerated the re-epithelialization from the edge.

Conclusion: Collectively, these data suggest that ADSC is constitutionally well suited for dermal wound healing and secretory factors derived from ADSCs promote wound healing via HDFs and ADSCs can be used for the treatment of photoaging and wound healing.

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1. Introduction

Adipose-derived stem cells (ADSCs) are a population of pluripotent mesenchymal cells, which can differentiate into various lineages [1–3]. Some clinical applications of ADSCs for compensation of diverse tissue defects have been suggested [4–6]. However, few studies have dealt with the effects of ADSCs on fibroblasts, which play a key role in skin biology such as wound healing, scar and photoaging. On the other hand, several lines of evidences reported that bone marrow-derived mesenchymal stem cells (BM-MSCs) and some growth factors accelerate wound healing [7–9]. ADSCs have surface markers and gene profiling similar to BM-MSCs. Given their convenient isolation compared with BM-MSCs and extensive proliferative capacities *ex vivo*, ADSCs hold great promise for use in wound repair and regeneration.

In a highly coordinated biological process of dermal wound healing, skin fibroblasts interact with surrounding cells such as keratinocytes, fat cells and mast cells. Fibroblasts produce extracellular matrix (ECM), glycoproteins, adhesive molecules and various cytokines [10]. By these syntheses and cell-to-cell, cell-to-cytokine interdependencies, skin fibroblasts contribute to the fibroblast–keratinocyte–endothelium complex that not only repairs wounds but also maintains the integrity and youth of skin. In the early phase of wound healing, fibroblasts migrate into the affected area and move across fibrin-based provisional matrix. Since the provisional fibrin-based matrix is relatively devoid of fibroblasts, the processes of migration, proliferation and ECM production are the key steps in the regeneration of a functional dermis [11]. Fibroblasts produce collagen-based ECM that ultimately replaces the provisional fibrin-based matrix and help re-approximate wound edges through their contractile properties.

In an attempt to explore the contribution of ADSCs to the cutaneous wound healing, we investigated whether conditioned medium of ADSCs (ADSC-

CM), together with cell-to-cell contact between ADSCs and human dermal fibroblasts (HDFs), promote proliferation. In addition, secretion of type I collagen was examined followed by measuring the mRNA levels of ECM proteins and effect on migration activity of HDFs was tested in *in vitro* wound healing model. As an alternative therapeutic strategy for damaged tissue, we studied the effect of ADSCs on wound size and skin re-epithelialization in animal model.

2. Materials and methods

2.1. Isolation and culture of ADSCs and HDFs

Human subcutaneous adipose tissue samples were acquired from elective liposuction of 23 healthy females with informed consents as approved by the institutional review boards. The obtained samples were digested with 0.075% collagenase type II (Sigma–Aldrich, St. Louis, MO) under gentle agitation for 45 min at 37 °C, and centrifuged at 300 × g for 10 min to obtain the stromal cell fraction. The pellet was filtered with 70 μm nylon mesh filter, and resuspended in phosphate-buffered saline (PBS). The cell suspension was layered onto histopaque-1077 (Sigma–Aldrich), and centrifuged at 840 × g for 10 min. The supernatant was discarded, and the cell band buoyant over histopaque was collected. Retrieved cell fraction was cultured overnight at 37 °C/5% CO₂ in control medium (Dulbecco's modified Eagle media (DMEM), 10% fetal bovine serum (FBS), 100 U/ml of penicillin, 100 μg/ml of streptomycin). The resulting cell population was maintained over 3–5 days until confluence, which were represented as passage 1. ADSCs were cultured and expanded in control medium, and used for the experiments at passages 1–5. Punch biopsy samples were obtained from the same donors for ADSCs, and HDFs were cultured as previously described [12].

2.2. Flow cytometric characterization of ADSCs

ADSCs of passage 3 cultured in a control medium for 48 h prior to analysis were incubated with monoclonal PE-conjugated antibodies for CD34 and CD73 (both BD Pharmingen, San Diego, CA), or with FITC-conjugated antibodies for CD49d and CD90 (Chemicon, Temecula, CA) and CD105 (BD Pharmingen) for 30 min at room temperature. As control, cells were stained with isotype control IgG. Cells were subsequently washed with PBS, fixed with 4% formaldehyde, and analyzed on FACScan flow cytometer (Beckton Dickson, San Jose, CA) using CELLQuest Pro software.

2.3. Contact co-culture of HDFs with ADSCs

Equal numbers of HDFs and ADSCs (1×10^5 cells) were allowed to attach to six-well culture dishes overnight, and were incubated in serum-free DMEM for 1 day to generate lawns with comparable cell confluences. HDFs were labeled with 5 μ M of Dil (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes, Eugene, OR) dye for 30 min, plated (3×10^4 well⁻¹) on HDF or ADSC lawns, and maintained in the culture medium with 2% FBS for 3 days. Proliferations of co-cultured HDFs on either HDFs or ADSCs were compared by their Dil red fluorescence with inverted fluorescence microscope (Olympus IX70, Tokyo, Japan) and FACScan analysis.

2.4. Transwell co-culture of HDFs with ADSCs

To verify the effect of soluble factors secreted by ADSCs on HDF proliferation, HDFs were co-cultured with ADSCs in two-chamber dishes preventing direct contact but permitting exchange of soluble diffusible factors. HDFs (1×10^4 cells/well) were seeded onto a polycarbonate membrane of transwell (0.45 μ m pores; collagen coated; Corning Costar, Cambridge, MA) with ADSCs lawns in the lower chambers, and cultured for 3 days in DMEM with 2% FBS. HDFs grown in upper chambers of transwell were fixed with methanol and stained with Hematoxylin and Eosin H&E. Cell images from five random fields per well were captured and counted.

2.5. Harvest, preparation, and ELISA of ADSC-CM

ADSCs (4×10^5 cells) were plated on 100 mm dish overnight with control medium, and were cultured in

DMEM/F12 (Invitrogen-Gibco-BRL, Grand Island, NY) serum-free medium. CM was collected after 72 h of culture, centrifuged at $300 \times g$ for 5 min, and filtered through 0.22 μ m syringe filter. Collected CM was applied to HDFs at varying dilution folds (0, 10, 50, and 100%) in DMEM/F12 with FBS concentration adjusted to 2%. The concentrations of several cytokines and ECM proteins were measured using sandwich ELISA kits (vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor (TGF)- β 1 and β 2, hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), platelet-derived growth factor (PDGF)-AA, placenta growth factor (PLGF), type I collagen, and fibronectin; R&D systems, Minneapolis, MN) according to the manufacturer's instructions.

2.6. Measurement of HDF proliferation in ADSC-CM

HDFs (1×10^3 cells/well) were plated in 96-well plates in DMEM with 0.1% FBS for 24 h to arrest mitosis. The media were then replaced by varying dilutions (0, 10, 50, and 100%) of ADSC-CM. After 72 h, HDF proliferation was measured using a CCK-8 kit (Dojindo, Gaithersburg, MD). HDFs were added 10 μ l of the CCK-8 solution, and incubated for 3 h, and then the absorbance was measured at 450 nm using a microplate reader (TECAN, Grödig, Austria). OD values of each well were calculated to their relative cell numbers with comparable standard curves.

2.7. Western blot of type I collagen secreted by HDFs

HDFs (2×10^4 cells/well) were seeded in 24-well plates and cultured overnight in DMEM with 0.1% FBS. Aliquots of culture media were separated using 8% sodium dodecyl sulfate-polyacrylamide gel, and blotted onto PVDF membranes (Millipore, Bedford, MA). Blots were incubated with type I collagen primary antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), washed, and incubated with a secondary antibody conjugated to horseradish peroxidase (1:10,000, Zymed, San Francisco, CA). Immunoreactive bands were detected with a chemiluminescent substrate system (immunobilon western reagent; Millipore), and quantified by densitometer and GeneTools software (Syngene, Cambridge, UK).

2.8. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

HDFs were exposed to ADSC-CM of varying concentrations (0, 10, 50, and 100%) for 4 h. Total cellular

RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) followed by a reverse transcription with cDNA synthesis kit (Promega, Madison, WI). cDNA was synthesized from 1 μ g total RNA using 200 U of reverse transcriptase (M-MLV RT) and 20 pM oligodT. The following oligonucleotides were used as primers: collagen type I (5'-tcaaggtttc-caaggacctg-3' and 5'-tcaaggtttccaaggacctg-3'), collagen type III (5'-aaaggggagctggctacttc-3' and 5'-gcgagtaggagcagttggag-3'), fibronectin (5'-tgaagag-gggcacatgctga-3' and 5'-gtgggagttgggctgactcg-3'), matrix metalloproteinase (MMP)-1 (5'-aaaatcctgtc-cagcccatcg-3' and 5'-ttctgtcctgaacagccagct-3'), and the internal control β -actin (5'-accctgaagtacc-catcg-3' and 5'-caccggagtccatcacg-3'). PCRs were performed in a final volume of 20 μ l reaction mix that contained 2 μ l of the RT reaction mixture, 15 mM MgCl₂, 1.25 mM dNTP, 20 pM of each primer, and 0.5 U of Taq polymerase (Promega). Thermal cycling over 30 times, consisted of an initial denaturation at 94 °C for 5 min, then 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and was terminated by a final extension at 72 °C for 5 min. β -Actin mRNA level was

used for sample standardization. After electrophoresis on 1% agarose gel, each band was quantified by densitometer and GeneTools software.

2.9. HDF migration assay

For the measurement of cell migration, confluent HDFs kept in serum-free medium for 24 h were wounded with a plastic micropipette tip having large orifice. After washing, the medium was replaced by control medium or varying concentrations of ADSC-CM. Photographs of wounded area were taken every 24 h by phase-contrast microscopy. For evaluation of wound closure, four randomly selected points along each wound were marked, and the horizontal distance of migrating cells from the initial wound was measured.

2.10. *In vivo* mouse wound healing model

Six female nude mice (Orient Bio, Sungnam, Korea) were used in animal experiments. Experimental protocols involving the mice used in this study were

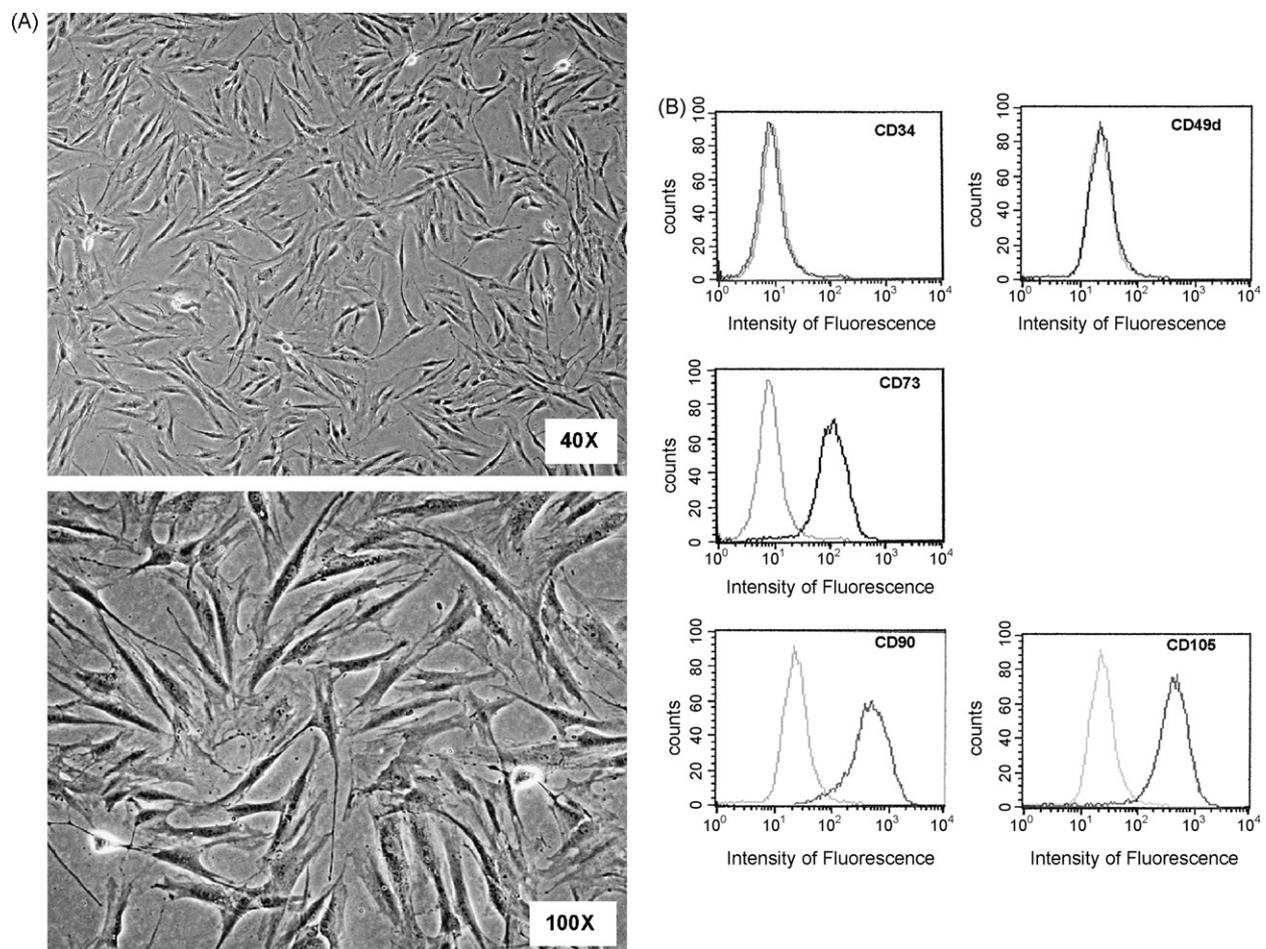


Fig. 1 Characterization of human ADSCs. Adherent and fibroblastic morphology of ADSCs (A). Flow cytometric histograms of human ADSCs (B). The gray line represents control and the solid line represents the specific antibodies indicated.

reviewed by the Animal Care and Use Committee in College of Pharmacy, Seoul National University according to the NIH guidelines (NIH publication number 85-23, revised 1985) of Principles of Laboratory Animal Care.

On the operation day, mice were anesthetized by subcutaneous injection of a mixture of ketamine (20 mg/kg) and acepromazine (10 mg/kg). Two circular full-thickness wounds of 7 mm diameter were created on the backs of mice. ADSCs were prepared before the experiment. Collagen gel solution (left side of the back) and collagen gel solution with 1×10^6 ADSC cells (right side of the back) were placed in the wounds and dressed with transparent Tegaderm[®] (3M Health Care, St. Paul, MN). Animal behavior and bandage integrity were monitored during the experiment (Fig. 7A). Wounds were evaluated 7 days after surgery. Digital pictures were taken to visualize the wound. Wound healing was quantitatively measured and calculated by the

remaining wound area. In addition, histological analyses using H&E staining of wounds were performed 21 days after surgery.

2.11. Statistical analysis

Data are representative of three or more independent experiments. One-way ANOVA test followed by paired *t*-test was used for statistical analysis and $P < 0.01$ or 0.05 was considered significant.

3. Results

3.1. Characterization of ADSCs

The average number of processed lipoaspirated (PLA) cells isolated was $2.4 \pm 1.4 \times 10^6$ cells ($n = 23$)/ml of lipoaspirated fat. ADSCs expanded easily *in vitro* by culturing PLA cells and exhibited

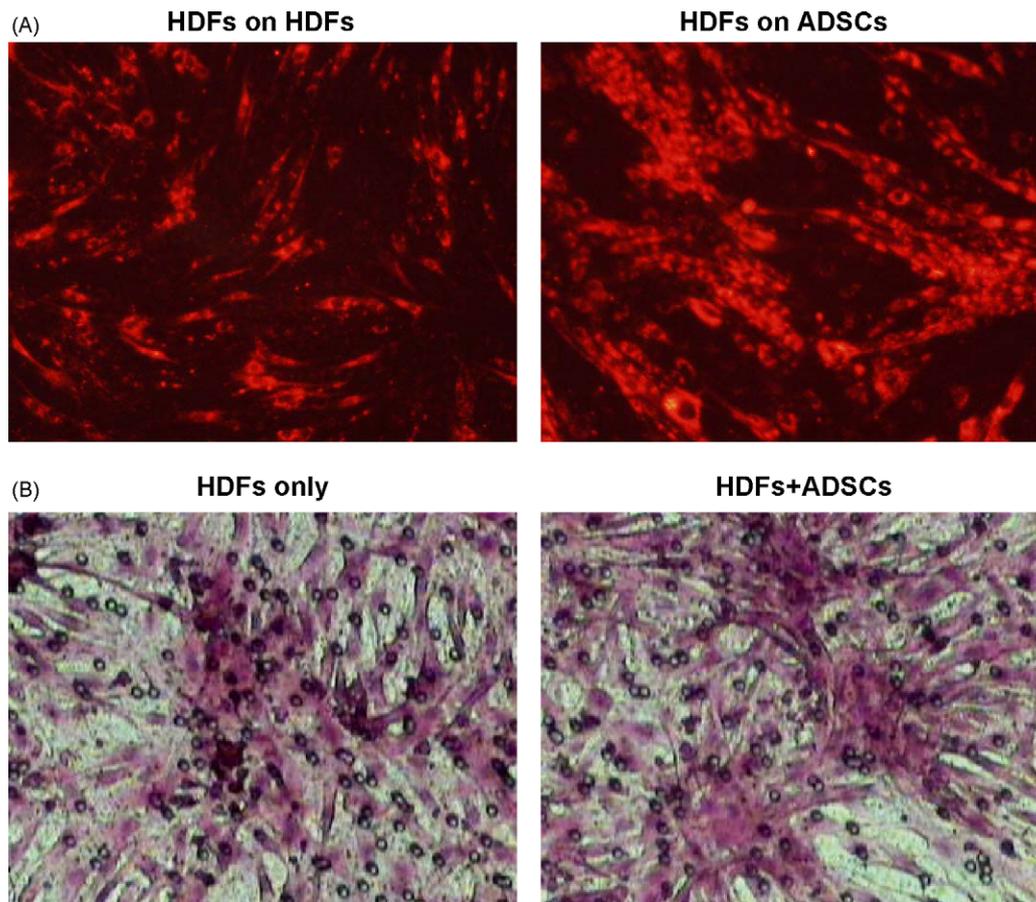


Fig. 2 Co-culture of HDFs with HDFs or ADSCs. Dil-labeled HDFs have grown for 2 days in contact with either HDFs (left) or ADSCs (right) lawns and visualized by inverted fluorescence microscope (A). The photographs shown are representative of four independent experiments. For co-culture without direct cell contact, HDFs were cultured on the permeable membrane (0.45 μm pores) of transwell chamber dishes with (right) or without (left) ADSCs. After 3 days, HDFs on the membrane of the upper chamber were stained with H&E (B). Results are shown as mean \pm S.D. of quadruplicate cultures within a representative of three experiments.

a fibroblast-like morphology (Fig. 1A). In flow cytometry, characteristic expressions of stem cell-related surface markers were confirmed. ADSCs expressed CD73, CD90, and CD105, and were lacking in CD34 and CD49d (Fig. 1B). Adipogenic, osteogenic, and chondrogenic differentiation was also confirmed by conventional method (data not shown).

3.2. Stimulatory effect of ADSCs on HDF proliferation

In direct co-culture experiments, stimulatory effect on HDF proliferation was compared between ADSCs and HDFs. HDFs were obtained from the same donor of ADSCs when co-cultured for each experiment. Flow cytometric analysis of DiI-labeled HDFs, revealed that ADSCs were superior to HDFs in promoting HDF proliferation (67.32% versus 30.08%) (Fig. 2A). In a transwell co-culture model, soluble factors secreted by ADSCs promoted HDF proliferation, although ADSCs were physically separated from HDFs (Fig. 2B). HDFs cultured in transwell with ADSC lawns showed significantly enhanced proliferation compared to control HDFs cultured without ADSC lawns ($119.9 \pm 7.5\%$ increase compared with control, $P < 0.01$).

3.3. ADSC-CM promotes proliferation of HDFs

Because the proliferation of fibroblasts is an important aspect of wound healing, we measured the effects of ADSC-CM on HDF proliferation. No obvious morphologic changes were observed in HDFs (Fig. 3A). ADSC-CM enhanced HDF proliferation in a dose-dependent manner (Fig. 3B), and the numbers of HDFs cultured for 3 days in 50 and 100% of ADSC-CM were significantly higher than that of HDFs in normal culture medium. The proliferative effect of ADSC-CM was completely abolished by boiling, suggesting that it was due to a specific receptor/ligand interaction (data not shown).

3.4. ELISA of secreted growth factors and ECM proteins released from ADSCs

ADSCs produced a variety of growth factors: PDGF (44.41 ± 2.56 pg/ml), PlGF (37.87 ± 1.69 pg/ml), basic FGF (131.35 ± 30.31 pg/ml), KGF (86.28 ± 20.33 pg/ml), TGF- β 1 and β 2 (103.33 ± 1.70 and 75.42 ± 95.98 pg/ml), HGF (670.94 ± 86.92 pg/ml), and VEGF (809.53 ± 95.98 pg/ml). In addition, we measured type I collagen and fibronectin among ECM proteins based on the proteomic analysis of proteins secreted by ADSCs (data not shown). The concentra-

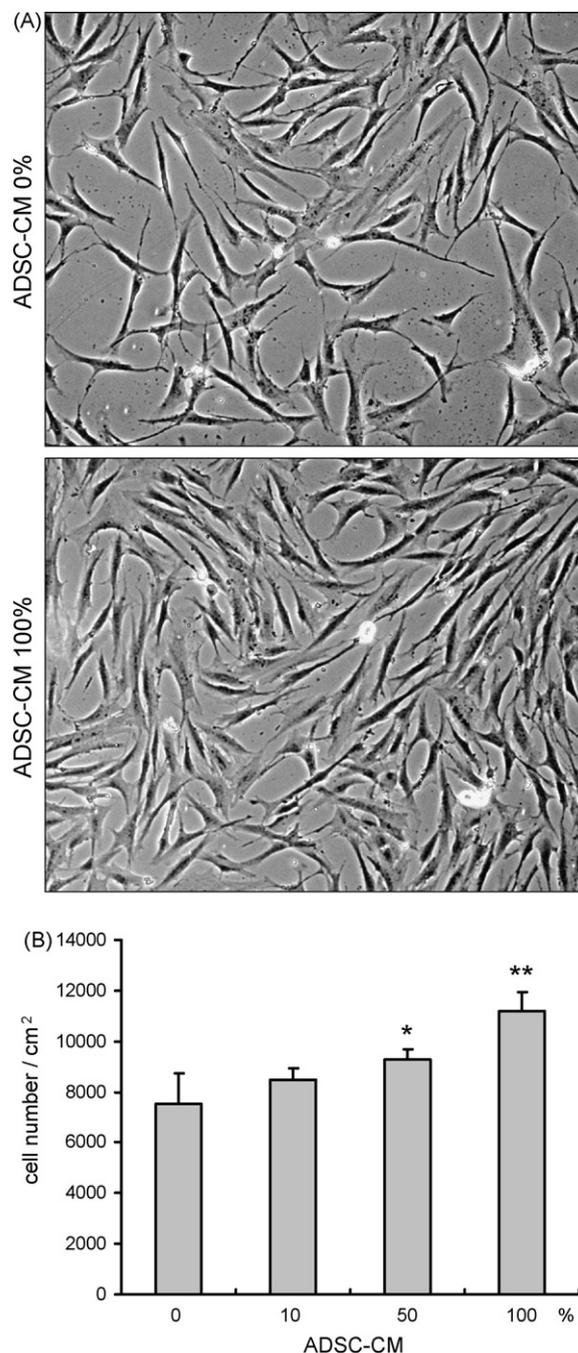


Fig. 3 Effects of ADSC-CM on proliferation of HDFs. HDFs were treated with 0, 10, 50 and 100% of ADSC-CM for 72 h. HDFs were exposed to control medium or 100% ADSC-CM (A). Numbers of HDFs exposed to ADSC-CM were measured by CCK assay (B). Results are shown as mean \pm S.D. of quadruplicates within a representative of three independent experiments. * $P < 0.05$; ** $P < 0.01$.

tions of type I collagen (921.47 ± 49.65 ng/ml) and fibronectin (1466.48 ± 460.21 ng/ml) were much higher than those of growth factors. Although we do not directly investigate the effect of respective growth factor on fibroblast proliferation, several

lines of evidences suggest that they play a pivotal role in wound healing process [8,13–15].

3.5. ADSC-CM increases type I collagen secretion by HDFs

In Western blot analysis, Fig. 4A and B shows that ADSC-CM has a more potent effect on type I collagen secretion by HDFs than does HDF-CM. In addition, ADSC-CM up-regulated the type I collagen synthesis by HDFs in a dose-dependent manner (Fig. 4C and D).

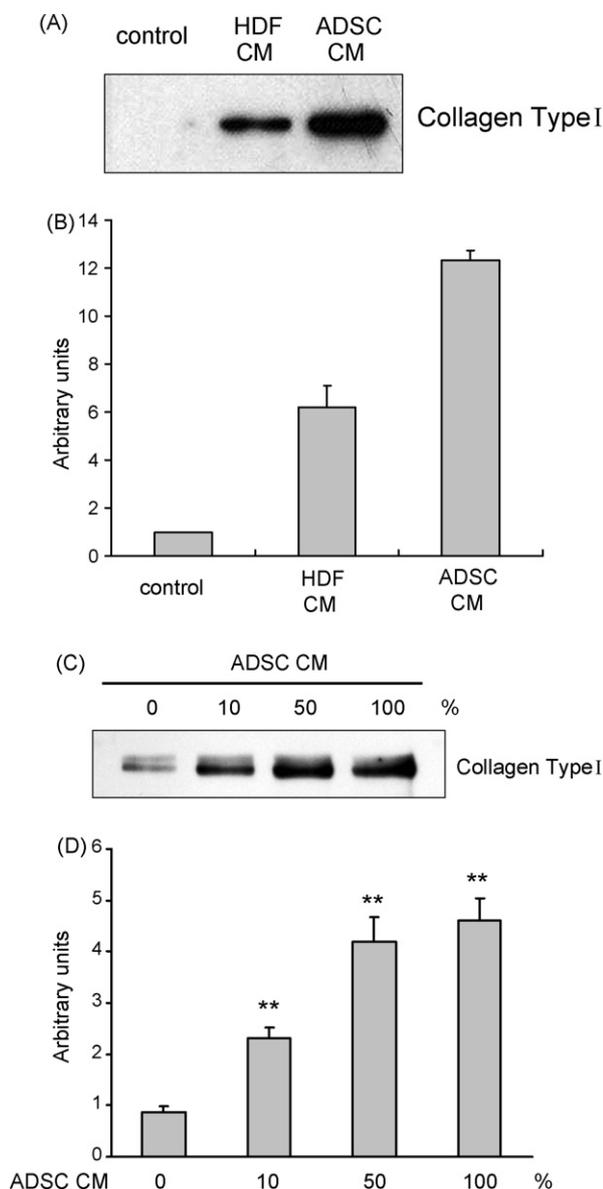


Fig. 4 Effects of ADSC-CM on the secretion of type I collagen by HDFs. Secretion of type I collagen was determined by Western blot analysis. ADSC-CM increased the type I collagen expression higher than did HDF-CM (A and B) and in a dose-dependent manner (C and D).

3.6. ADSC-CM regulates the mRNA expression of ECM proteins in HDFs

In order to assess ADSC-CM-induced modulations of mRNA expression level, RT-PCR was performed on types I and III collagen, fibronectin, and MMP-1. ADSC-CM up-regulated the mRNA expression of type I collagen ($P < 0.05$), while that of β -actin remained unaffected. mRNA expressions of type III collagen and fibronectin were also up-regulated (both $P < 0.01$), while that of MMP-1 was down-regulated ($P < 0.01$) (Fig. 5A). Dose–response relationship of mRNA expression of ECM proteins was also demonstrated (Fig. 5B).

3.7. ADSC-CM promotes HDF migration

To examine whether ADSC-CM exhibited biological effects relevant to migration of HDFs, wound healing migration assay were performed. In wound healing migration assay, *in vitro* migration of HDFs was

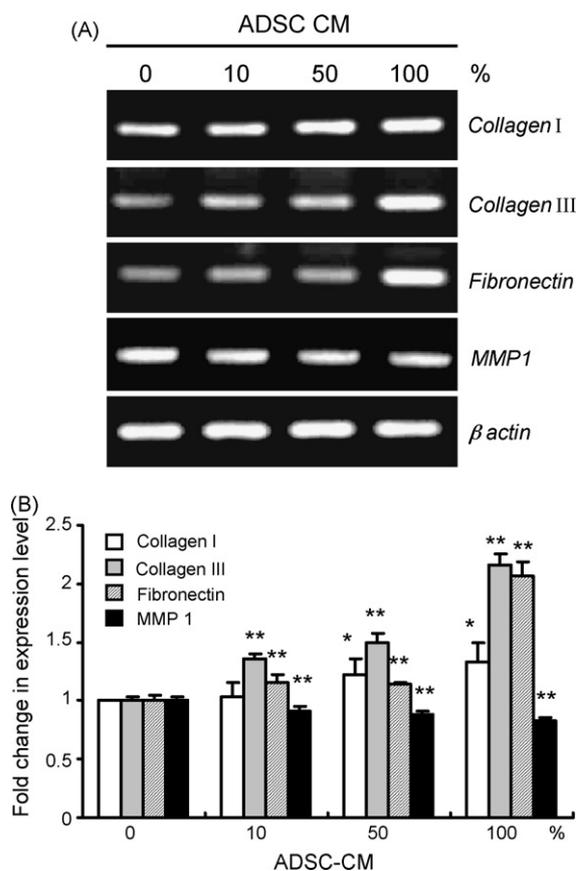


Fig. 5 Effects of ADSC-CM on mRNA expressions of ECM proteins and MMP-1 in HDFs. mRNA expression of types I and III collagen, fibronectin and MMP-1 were detected by RT-PCR (A) and estimated compared with β -actin (B). Representative data are shown as mean \pm S.D. of triplicates within a representative of four independent experiments. * $P < 0.05$; ** $P < 0.01$.

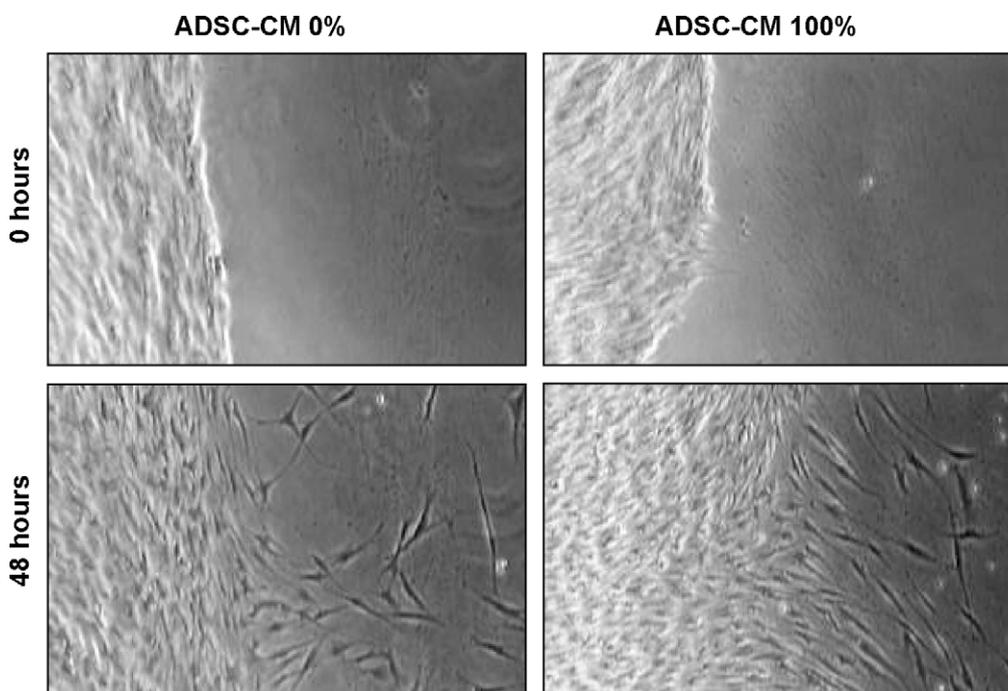


Fig. 6 Effects of ADSC-CM on HDF migration. Monolayers of confluent HDFs were wounded with plastic micropipette tips. HDFs were photographed 48 h after wounding by phase-contrast microscopy.

determined by wounded spaces on culture plates. ADSC-CM enhanced migration of HDFs by 1.97-fold over control level ($P < 0.01$) after 48 h incubation (Fig. 6).

3.8. ADSCs promote wound healing *in vivo*

In a preliminary study, we directly compared ADSC treatment with collagen gel solution and without any transplantation, and verified that ADSCs in collagen gel solution are effective in wound healing (data not shown). To confirm the practical role of ADSCs, treatment of collagen gel solution and that of ADSCs in the collagen gel solution was tested. Compared with the collagen gel treatment (left side of the back), ADSCs treatment significantly accelerated wound healing in the right side of the back after 7 days (Fig. 7B). In addition, the beginning of re-epithelialization from the wound edge was clearly observed in the wounds treated with ADSCs. ADSC treatment reduced the wound areas and reduced area was quantitatively measured in Fig. 7C ($34 \pm 15\%$ reduction in the ADSC-treated group, $P < 0.05$, $n = 6$). Wound closure was much faster in the ADSC-treated group than in the control group. Closure was almost achieved within 14 days. H&E staining of wounded skins were performed in 21 days after surgery, but no significant difference of skin structure was found between the slices of control and ADSC treatment (Fig. 7D). In addition, no significant dermal inflammatory cell infiltration was found in ADSC-treated mice. It seems that ADSCs

accelerate wound healing without an abnormal wound healing process, such as granulation or epidermal hyperplasia.

4. Discussion

In this study, ADSCs were superior to HDFs in promoting HDF proliferation and ADSC-CM was also superior to HDF-CM in up-regulating type I collagen secretion by HDFs (Figs. 2A and 4A). In our preliminary proteomic analysis and other reports [16,17], ADSCs secrete several collagens, fibronectin and growth factors. The concentrations of type I collagen and fibronectin were found to be at least 1000-fold higher than those of several growth factors. In addition, ADSCs promote the mRNA expression of ECM proteins and HDF migration. In animal model, ADSCs significantly reduced the wound size and accelerated the re-epithelialization from the edge. Collectively, these data suggest that ADSCs are constitutionally well suited for dermal wound healing and secretory factors derived from ADSCs promote wound healing via HDFs.

In contact co-culture experiment, there was no difference in the attachment of Dil-labeled HDFs on both HDF and ADSC lawns 12 h after seeding, suggesting that HDF proliferation might not be affected by the efficiency of initial attachment to the underlying lawns. The reproduction of the mitogenic effect of ADSCs in transwell co-culture (Fig. 2B)

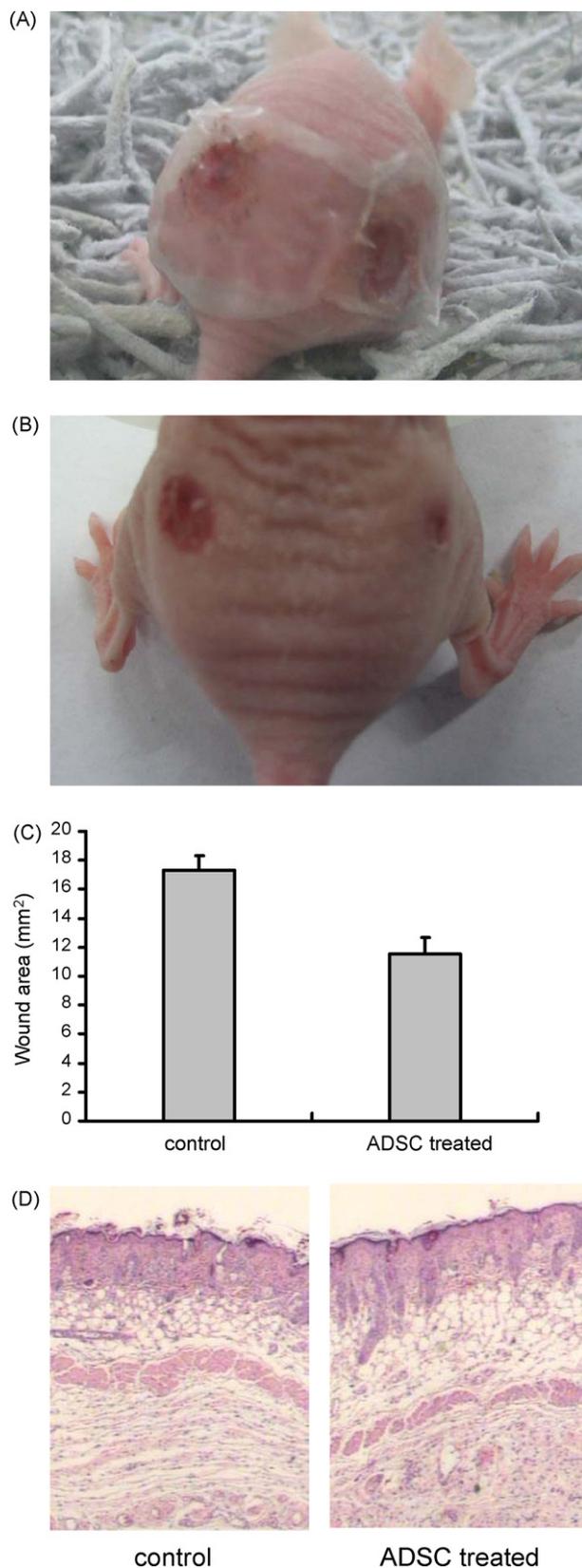


Fig. 7 Wound healing effect of ADSCs in nude mice. Animal behavior and bandage integrity were monitored from 1 day after surgery (A). Wound size reduced significantly in the ADSC-treated group (right side of the back)

and ADSC-CM (Fig. 3), may be caused by the action of at least several cytokines or ECM proteins, although the contribution of the synergy or unknown substances was not elucidated in this study. Our examination of secreted soluble factors in ADSC-CM showed that ADSCs produce various growth factors such as PDGF, insulin-like growth factor (IGF), KGF in addition to the previously reported growth factors: bFGF, TGF- β , HGF, and VEGF [18]. These cytokines do not operate in isolation but rather interact with other regulatory proteins. No single growth factor could reproduce the activity of ADSC-CM and combination of several growth factors, ECM proteins or other unknown substances might account for the full effect of ADSC-CM. For fibroblasts, the main implicated cytokines are PDGF, latent and active TGF- β 1, interleukin (IL)-1 β , FGFs, IL-6, and IL-10 [19]. Among these, PDGF and TGF- β may play a key role in wound healing process [8,20].

Several researches on the pathophysiology of photoaging have found correlations with certain aspects of wound healing. Histologic features of photoaged skin show marked alterations of ECM composition. The collagenous component of dermal ECM is responsible for the strength and resiliency of skin and is intimately involved in the pathology of photoaging. In photoaged human skin, the precursors of fibrillar types I and III collagens are significantly reduced in the papillary dermis and their reduction has been shown to correlate well with clinical severity of photoaging. This reduction results from a combination of reduced procollagen biosynthesis and increased enzymatic breakdown via the actions of MMPs.

In summary, we investigated the interaction between ADSCs and HDFs for the first time, as the application of ADSCs for dermal wound healing remained speculative. ADSCs have effects on HDFs by increasing collagen synthesis and by activating proliferation and migration activity of HDFs, suggesting that ADSCs or ADSC-CM can be used for the treatment of photoaging and wound healing. Our results also suggested that the ADSCs are constitutionally well suited for dermal wound healing compared with HDFs. Mainly with secreted growth factors or ECM proteins, ADSCs contribute to enhanced wound healing potential of HDFs. Further mechanism studies using neutralizing antibodies against each growth factor, may clarify the role of soluble factors of ADSC in wound healing process.

compared with the control after 7 days (B). Reduced wound area was measured 7 days after surgery (C) and histological analysis of repaired skin was stained with H&E in 21 days after surgery (D).

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