

Adipose-derived stem cells accelerate neovascularization in ischaemic diabetic skin flap *via* expression of hypoxia-inducible factor-1 α

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Abstract

Skin flaps are frequently performed for diabetic patients in spite of countless detrimental effects of diabetes on flap survival, most of which may result from a defective response of the tissues to low oxygen tension. In this study, the authors explored the feasibility of applying human adipose-derived stem cells (ASCs) to increase the viability of random-patterned skin flaps in streptozotocin-induced diabetic mice. ASCs were isolated from the fresh human lipoaspirates and expanded *ex vivo* for three passages. After the elevation of caudally based random-patterned skin flaps (3 cm long and 1 cm wide), ASCs suspensions were then injected into the flap (group A). Media containing no ASCs were similarly injected as a control (group B), although nothing was injected into the flap base of mice in control group C. Flap assessments were carried out at post-operative day 7 for evaluation of flap viability. The flap survival rate of group A was significantly higher than those of groups B and C, whereas no difference was observed between groups B and C. Histological examination also demonstrated a statistically significant increase in capillary density in group A over both groups B and C. Furthermore, it was found that ASCs not only augmented the expression of vascular endothelial growth factor and hypoxia-inducible factor-1 α (HIF-1 α) in flap tissues from dermis of diabetes mice, but also promoted their expression in dermal fibroblasts from diabetic mice. Thus, ASCs could enhance the survival of random-patterned skin flaps in streptozotocin-induced diabetic mice *via* elevated expression of HIF-1 α .

Keywords: diabetes mellitus • adipose-derived stem cells • skin flaps

Introduction

As a complex metabolic disease, diabetes mellitus (DM) is estimated to be involved with 171 million adult cases worldwide [1], among which impaired healing of foot ulcers occurs frequently and represents the most prevalent cause of lower extremity amputation [2]. For treatment of these chronic wounds in diabetic patients, random skin flap has been widely used in clinic. However, diabetic skin flap exhibits a higher rate in partial or complete flap necrosis than non-diabetic flap does, which is a common problem encountered post-operatively [3–5]. Poor healing of diabetic wounds and necrosis of diabetic skin flap have been attributed to deficient ischaemia-driven neovascularization under hypergly-

caemia conditions [6]. Moreover, accumulative evidence suggests that impaired neovascularization is a consequence of diminished levels of pro-angiogenic factors production in diabetes. Thus, how to increase pro-angiogenic factor levels, and thereby augmenting angiogenesis, plays an essential role in improving the survival of diabetic skin flap.

Existing evidence has suggested that adipose-derived stem cells (ASCs) may be useful for improving survival of diabetic skin flap [7–9]. In addition to their multiple differentiation potential [10, 11], *in vitro* cultured ASCs have been documented to secrete a variety of growth factors such as PDGF, TGF- β and VEGF, which are known to favour angiogenesis, extracellular matrix (ECM) deposition as well as tissue remodelling in healing of injured wound [9, 12]. It was reported that ASCs are able to stimulate proliferation, migration and matrix production of cultured dermal fibroblasts (DFs) *via* a paracrine pattern [13]. More importantly, potential of these *in vitro* favourable characteristics of ASCs to facilitate healing of cutaneous wound has been documented by several *in vivo* studies [12, 14]. Altman *et al.* [15] observed that human

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ASCs seeded onto decellularized dermal scaffolds can improve healing in wounds of nude mice. Recently, the effectiveness of ASCs in improving viability of skin flap in non-diabetic mice has been demonstrated in a study by Lu *et al.* [7], in which they injected ASCs at flap pedicle locally and found that a nearly two-fold increase in surviving area was achieved when compared with flap without ASCs administration. Furthermore, emerging works [16] showed that healing of diabetic wound can also be improved by implantation of ASCs. However, it is still unclear whether topical application of ASCs could improve viability of diabetic skin flap, which represents a more complicated pathophysiological situation and lacks effective intervene approach clinically.

According to previous studies, the enhanced neovascularization in ischaemic tissue by introduction of mesenchymal stem cells (MSCs) have been mainly attributed to two distinct mechanisms. First, engrafted MSCs produced high levels of angiogenesis stimulating factors such as VEGF, PDGF and bFGF, to improve neovascularization *in situ* through paracrine pathway. Secondly, implanted MSCs have been observed to participate into blood vessels with acquirement of endothelial cell phenotype, thereby augmenting blood supply by improving capillary density. As a master transcription factor that is essential for adaptive responses of the cell to hypoxia, hypoxia-inducible factor-1 α (HIF-1 α) is necessary for expression of multiple angiogenic growth factors [17, 18], cell motility [19] and their incorporation into blood vessels [20]. Studies have shown that hyperglycaemia impairs the stability and function of HIF-1 α , resulting in the suppression of expression of HIF-1 target genes essential for neovascularization [21]. Thus, it is of importance to understand whether topically administered ASCs could correct HIF-1 α expression under hyperglycaemic circumstances and thereafter, improve neovascularization in ischaemic diabetic skin flap.

In this study, we hypothesize that topically injected ASCs may improve viability of ischaemic random skin flap in streptozotocin (STZ)-induced diabetic mice. We then assessed expression of angiogenic growth factors and localization of introduced Dil-labelled ASCs within flap tissues to investigate by which way the engrafted ASCs stimulated neovascularization. Finally, expression of HIF-1 α in skin flap and in ASCs cultured in high glucose (HG) under exposure to hypoxia was determined.

Materials and methods

Harvesting and expanding of human ASCs and mouse dermal fibroblasts (mDFs) *in vitro*

Fresh human lipoaspirates were obtained from three healthy patients (at an average age of 33 years) who had received abdominal liposuction at the Department of Plastic and Reconstructive Surgery of Shanghai 9th People's Hospital. All protocols of human tissue handling were approved by the Research Ethical Committee of the Hospital. Processed lipoaspi-

rate (PLA) cell isolation and culture were performed as previously described [22]. In brief, lipoaspirates were washed intensively with an equal volume of 0.1 M phosphate buffered saline (PBS, pH 7.4) and were digested with 0.075% collagenase type I (Washington glucose DMEM, LG-DMEM; Gibco, New York, NY, USA), containing 10% foetal bovine serum (FBS; HyClone, Logan, UT, USA), and the digested lipoaspirates were centrifuged at $1200 \times g$ for 10 min. to obtain a high-density stromal vascular fraction (SVF). Then the SVF collection was treated with red blood cell lysing buffer (0.3 g/l ammonium chloride in 0.01 M Tris-HCl buffer, pH 7.5; Sigma-Aldrich, St. Louis, MO, USA) for 5 min., centrifuged at $600 \times g$ for 10 min. and then filtered through a 100-mm nylon mesh to remove undigested tissue. Cells were resuspended in LG-DMEM culture medium, containing 10% FBS, 100 mg/ml streptomycin, 100 U/ml penicillin (growth medium), and plated at 4×10^4 cells/cm² in $\varnothing 100$ mm culture dishes (Falcon, USA) with the medium changed twice a week. When reached 70–80% confluence, cells were passaged and ASCs before passage 4 were used in the following study.

Primary isolates of DFs were harvested from STZ-induced diabetic mice and non-diabetics for each experiment, respectively. The animals were sacrificed and trunk skin was removed by sharp dissection. Special care was taken to remove the underlying adipose tissue. The harvested skin was then minced and digested for 2 hrs in 0.10% collagenase I solution in serum-free low glucose (LG, 5 mM D-glucose) or HG (25 mM D-glucose) DMEM at 37°C. The dissociated cells were then centrifuged and resuspended in an LG or HG DMEM with 10% FBS and 1% antibiotic/antimycotic supplement. The cells were grown at 37°C with 100% humidity in 5% CO₂ in air. Medium was changed every other day, and cells were passaged before they reached 70–80% confluence. Experiments were performed with cultured cells at passage 3.

Flow cytometry analysis of human ASCs surface marker

Flow cytometry analysis of cell surface markers was performed as previously reported [23]. For flow cytometry analysis, ASCs at passage 3 were harvested and washed in flow cytometry buffer (FCB, 1 \times PBS, 2% FBS, 0.2% Tween-20). The identity of ASCs was performed respectively by incubating cell aliquots (1×10^6 cells) for 30 min. with fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated monoclonal antibodies: CD29-PE, CD44-FITC and CD105-PE. The absence of haematopoietic or endothelial cell contamination was also confirmed by the expression analysis with CD34-FITC, CD45-PE and CD31-PE. Flow cytometry analysis was performed on a FACs alibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Murine model of diabetes and random pattern skin flap

Forty-five BALB/c-nu/nu male mice aged 7–8 weeks and weighing 20–25 g were purchased from SLAC National Rodent Laboratory Animal Resources (Shanghai, China). An institutional review committee of Shanghai Jiao Tong University School of Medicine approved all animal study protocols. Mice were maintained under specific pathogen-free conditions (SPF). The animals were randomized into three groups of 15 mice each. DM was induced by intraperitoneal injections of 150 mg/kg STZ (Sigma-Aldrich) in citrate buffer (pH 4.5). A sample of the mouse's venous blood in the tail was collected on a reagent strip 14 days after the diabetes induction

procedure for blood glucose level determination using One Touch Ultra™ portable glucose analyser (Lifescan, Inc., Milpitas, CA, USA). Fourteen days after the initial injection, mice with blood glucose levels above 300 mg/dl were deemed diabetic. Only the animals with a blood glucose level above 300 mg/dl were used in the study.

Fourteen days after induction of diabetes, all surgical procedures were performed by only one surgeon by atraumatic technique. All procedures were conducted in accordance with the guidelines set forth by the Ethics Committee of Shanghai Jiao Tong University School of Medicine. All mice were anaesthetized with intraperitoneal injections of pentobarbital sodium (20 mg/kg body weight). During the surgical procedure, asepsis was maintained by providing a local sterile environment at SPF animal lab of Shanghai ninth people's hospital affiliated to Shanghai Jiao Tong University School of Medicine. After adequate anaesthesia depth was confirmed with the pinch flexion/withdrawal test, the animals were positioned over a flat surface with limbs extended. A random pattern, caudally based dorsal flap was performed in the dorsum of the mice by means of a transparent plastic pattern cut in standard dimensions (1.0×3.0 cm). The flap was then incised with scalpel, being elevated in a plane superficial to the deep muscular fascia, including the superficial fascia, panniculus carnosus, subcutaneous tissue and skin. No axial vessels were incorporated into the flap to produce a random pattern skin flap in which the ischaemic gradient is proportional to the distance from the base.

Dil-labelling and transplantation of ASCs

Cells were labelled with fluorescent Dil (V-22885, Molecular Probes, Eugene, OR, USA) according to the manufacturer's recommendations. Briefly, 5 μ l of the cell-labelling solution was added per millilitre of cell suspension (1×10^6 cells/ml). The suspension was then mixed well by gentle pipetting and followed by incubating at 37°C for 15 min. The labelled suspension in tube was centrifuged at 1500 rpm for 5 min. The supernatant was then removed and cells were gently resuspended in PBS for three times. Finally, PBS was removed and cells were gently resuspended in medium for transplantation.

For cell transplantation, the flap was first labelled with equant 10 sites at the media longitudinal axis on the flap as the respective injecting sites. After elevation of the flap, the mice in the group A ($n = 12$) received an injection of 1×10^7 Dil-labelled ASCs suspended in 100 μ L of serum-free LG DMEM into the flap. The mice in the group B ($n = 11$) received an injection of 100 μ L medium and the mice in the group C ($n = 12$) received nothing were both served as control groups. With meticulous haemostasis, the flap was sutured back into place with 5-0 running nylon sutures. The mice were given food and water *ad libitum*, and each mouse was maintained in its own cage.

Assessment of the survival areas of flaps

At day 7 after the operation, the surviving flap area was measured by digital image analysis. Pictures of the flaps at the same distance and on the same focus were taken by a digital camera. The survival of flaps was assessed by two specialists blindly with respect to the gross appearance, colour and consistency of the flaps and the presence or absence of cutting bleeding. The surface area of these defined zones was measured using Image-Pro Plus version 6.0 Software (version 6.0; Media Cybernetics LP, Silver Spring, MD, USA). The results were expressed as percentages of survival area relative to the total flap surface area.

Blood perfusion of the flap

In 5 mice from each group, tissue blood perfusion of skin flap was measured with laser Doppler flowmetry (Periflux system 5000, Sweden) at post-operative day 7 by two specialists blindly. All mice were anaesthetized with intraperitoneal injections of pentobarbital sodium (20 mg/kg body weight). The probe was placed on the proximal of necrosis line 0.5 cm from the distal necrosis edge of the flap for at least 30 sec., and the results were recorded as blood perfusion unit (PU). During measurement of blood flow, room temperature was maintained at around 24°C.

Histological assessment of flap

Tissue sections from the similar position of the flaps were harvested at 7 days post-operatively. Tissue specimens were fixed in 4% paraformaldehyde for 24 hrs and embedded in paraffin. Six- μ m-thick sections were stained with haematoxylin and eosin for light microscopy. Tissue specimens were embedded in optimum cutting temperature compound for frozen sections. Capillary density was assessed morphometrically by examining three fields per section of the flap in six successive sections from both the haematoxylin and eosin sections and the immunofluorescence staining sections after immunofluorescent staining for endothelial cells with an anti-CD31 antibody. For immunofluorescent staining, frozen cross-section of samples were fixed in cold acetone and stained with rat monoclonal anti-CD31 antibody (1:50, ab7388; Abcam Ltd., Hong Kong) primary antibodies, followed by addition of FITC-conjugated secondary antibodies (1:1000, A-11006; Invitrogen, Carlsbad, CA, USA). Nuclei were stained with Hoechst 33258 (Sigma-Aldrich).

Serial 6- μ m-thick sections were taken to detect Dil-labelled ASCs distribution inside flaps. On one hand, neovascularization was assessed by measuring the number of capillaries in 20 fields in each mouse in each group on haematoxylin and eosin stained slides [capillaries/high power field (HPF)]. On the other hand, vascular density was assessed by measuring the number of CD31⁺ cells on frozen sections. All the measurements were performed by two blinded reviewers.

Cell proliferation and migration assay

ASCs were grown in LG or HG and exposed to normoxia or hypoxia. Cell numbers were determined by DNA assay as our previously reported. Briefly, the cells collected at different time points were full lysed with proteinase K (0.5 mg/ml; Sigma-Aldrich) at 56°C overnight. The resulting mixture was subjected to centrifugation and aliquots (40 μ l) of the supernatants after being mixed with 160 μ l Hoechst 33258 dye solution (0.1 g/ml; Sigma-Aldrich) were transferred to black flat-bottomed 96-well plates (Corning Costar, New York, NY, USA). DNA content was quantified spectrofluorometrically using a Varioskan multimode detection reader (Thermo Electron Corp., Marietta, OH, USA) at a wavelength of 465 nm (the emission wavelength of 360 nm) by correlating with a DNA standard curve that was generated by lysing serial dilutions of a known concentration of ASCs.

The measurement of cell migration was performed as previously reported [24]. Cells were grown in LG or HG and exposed to normoxia or hypoxia in a humidified incubator at 37°C with 5% CO₂ until 85% confluence was reached. A wound was made by scratching the monolayer culture using a sterile 200 μ l micropipette tip. The monolayer culture was then washed four times with PBS (pre-warmed to 37°C) to remove the

floating cells, and after that 1.5 ml of culture medium was added. Photographs of wounded area were taken immediately and 24 hrs later by inverted light microscope. For evaluation of wound closure, four randomly selected points along each wound were marked, and the invaded/migrated area of each sample was calculated in comparison to that of time 0.

Transwell cultures

To verify the effect of soluble factors secreted by ASCs on diabetic mouse DFs, diabetic mouse DFs were cocultured with ASCs in transwell chambers with a 0.4- μ m pore size membrane (Millicell) using HG medium. Mouse DFs (1×10^4 cells/well) were seeded onto upper transwell chamber with ASCs in the lower chamber. After 2 days of coculture under normoxia, Cells were then placed in either normoxia (21% O₂) or hypoxia (1% O₂) for 24 hrs. Diabetic mouse DFs grown in upper chambers were harvested. Cell lysates were collected for Western blot of VEGF and HIF-1 α .

ELISA assay of VEGF production

ELISA was performed with sandwich ELISA kits according to the manufacturer's instructions: human VEGF was obtained from R&D systems (DY293B). Cell culture supernatants were used following standardization of each sample by total protein content using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Results are representative of four independent experiments.

Western blot analysis

Flaps or cells were processed by extracting proteins with a lysis buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS and 0.1% deoxycholate). Proteins were separated by 8% polyacrylamide gel electrophoresis containing 0.1% SDS and subsequently transferred to nitrocellulose membrane. The nitrocellulose sheet was blocked with 2% non-fat dry milk and 3% BSA in Tris-buffered saline. The polyclonal antibodies, rabbit anti-HIF-1 α (NB100-134; Novus Biologicals, Littleton, CO, USA), rabbit anti-VEGF (ab46154; Abcam Ltd., Hong Kong) and rabbit anti-SDF-1 α (5388-100; Biovision, Mountain View, CA, USA) were applied. The blots were developed using an IRDye 700DX- and IRDye 800CW-conjugated secondary antibody (Rockland Immunochemical, Inc., Gilbertsville, PA, USA), and proteins were visualized by the Odyssey system (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

One-way ANOVA was used to compare means for the three groups simultaneously. Furthermore, *post hoc* tests were performed to examine the difference between any two groups using least significant difference or Tamhane method when the variances were not homogenous across the groups. Differences were regarded as statistically significant for two-tail values of $P < 0.05$. All data were analysed using SPSS for Windows version 11.5 (SPSS, Inc., Chicago, IL, USA) and are presented as mean \pm S.E.M.

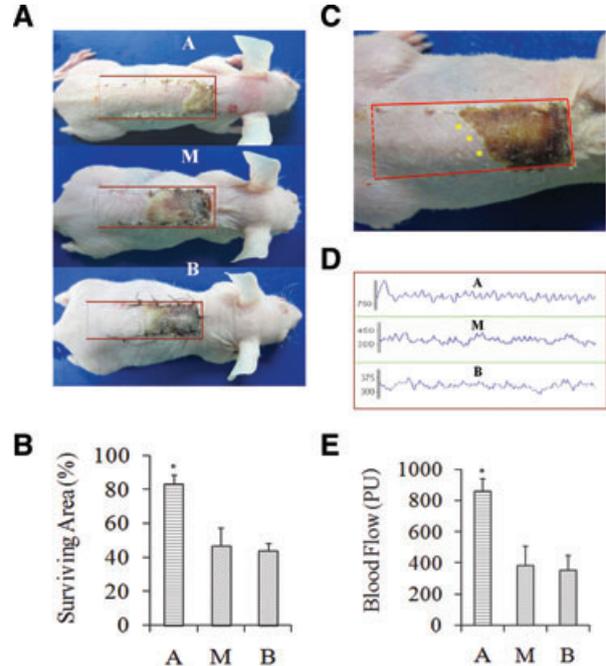


Fig. 1 The survival area and microvascular blood flow of random-patterned skin flap in diabetic mice 7 days after flap elevation. **(A)** Survival area of ischaemic tissue assessed at 7 days after surgery. **(B)** Mean survival percentages of flaps among three groups. $n = 8$, $P < 0.01$ versus M and B groups, respectively. **(C)** Flap microvascular blood flow was measured using laser Doppler velocimetry. The measurements were performed 0.5 cm from the distal necrosis edge of the flap in nine mice. **(D)** Representative examples of microvascular blood flow measurement of three groups. Segments of 30 sec. are displayed. **(E)** Mean blood flow perflusion of flaps of three groups. $n = 9$, $P < 0.01$ versus M and B groups, respectively. A: ASCs-treated group; M: medium-treated group; B: blank.

Results

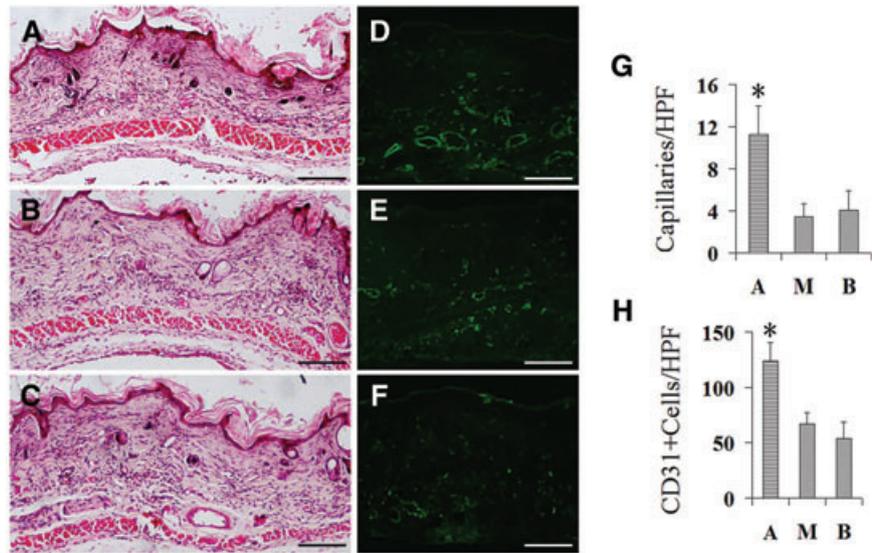
Characterization of human ASCs expanded *ex vivo*

After expansion to three passages, the cells were detached. It was found that cultured ASCs were positive for CD105 (48.55%), CD44 (97.57%), CD29 (66.28%) according to the flow cytometry analysis. In addition, the cell population was not contaminated by haematopoietic stem cells or endothelial cells, as demonstrated by flow cytometry analysis which showed that cells were negative for the CD31 (0.48%), CD34 (0.56%), CD45 (0.18%) antigens (data not shown).

ASCs improved flap viability *via* increasing blood perfusion

The surviving area in the flap was clearly distinguishable from necrotic one at day 7 post-operation in each group (Fig. 1A). By

Fig. 2 Microphotographs representing the comparative results of vascular density in the flaps of three groups. (A–C) Haematoxylin and eosin stains of the distal portion of flaps at 7 days post-operation of ASCs, medium- and blank-treated groups, showing increased neovascularization in the flap tissue of the ASCs group. (D–F) Frozen sections (8 μm) from the distal portion of random cutaneous flap tissue of three groups were stained with anti-CD31 antibody. The experiment was repeated three times, and one representative result is shown. (G) The number of blood vessels per high power field (HPF) was counted. $n = 9$, $*P < 0.01$ versus M and B groups, respectively. (H) The number of CD31-positive cells per HPF was counted. $n = 9$, $*P < 0.01$ versus M and B groups, respectively. Scale bars: 100 μm . A: ASCs-treated group; M: medium-treated group; B: blank.



quantitative digital analysis, the mean proportion of flap viability in group A (ASCs transplantation group), group B (culture medium injection group) and group C (control group) was $83.2 \pm 5.3\%$, $47.0 \pm 10.5\%$ and $43.7 \pm 4.5\%$, respectively (Fig. 1B). The surviving area in group A was significantly higher than that either in group B ($P < 0.01$) or in group C ($P < 0.01$). However, no significant difference was found between group B and group C ($P > 0.05$).

To study whether the improved viability in ASCs-treated flap (group A) was a result from the increase in blood supply, blood perfusion of flap was evaluated (Fig. 1C and D). The blood perfusion units in group A was 863.26 ± 76.52 , which was significantly higher than that either in group B (382.52 ± 125.64 , $P < 0.01$) or in group C (356.31 ± 93.91 , $P < 0.01$). However, no statistical difference was detected between groups B and C ($P > 0.01$) (Fig. 1E).

ASCs accelerated angiogenesis in ischaemic skin flap

As reported that ASCs could improve the survival of ischaemic skin flap through accelerating revascularization, we then investigated capillary densities in ischaemic skin flap of diabetic mice as shown in Figure 2. Histological observation of the flap tissue, which was obtained just adjacent to the necrotic boundary in each group, showed characterizations of normal skin with intact epidermis and dermis (Fig. 2A–C). However, samples in group A displayed an increased distribution of capillary vessels, which exhibited dilatant lumina as compared with vessels in groups B and C (Fig. 2A–C and G). To further investigate neovascularization in skin flaps, capillary densities at 7 days were assessed morphometrically by immunofluorescent staining for CD31 (Fig. 2D–F and H). Number of CD31⁺ cells was significantly higher in ASCs-treated

group than that either in medium-treated group or in control flaps group (Fig. 2H).

To determine whether transplanted ASCs incorporated into vessels wall, by which angiogenesis was enhanced, we pre-labelled cells with fluorescent CM-Dil before transplantation. The distribution of labelled cells was observed on day 7 (Fig. 3). The survival of transplanted ASCs was demonstrated by the presence of labelled cells, most of which localized subcutaneously in a pattern of aggregation close to vasculature. However, little labelled cells could be detected in the wall of blood vessels.

Several previous studies reported that cellular migration in diabetic wounds was severely impaired. Hence, to study whether impaired cellular migration is responsible for the failure of ASCs in participating into blood vessel wall in diabetic flap, migration assay of ASCs cultured under HG and hypoxic circumstances was performed (Fig. 4A and B). In normoxia conditions, migration of ASCs grown in HG medium was similar to those grown in LG without significant difference ($P > 0.05$). However, exposure to hypoxia for 24 hrs resulted in a remarkable increase in migration of ASCs, either cultured at low or HG levels, compared to the respective ones at the normoxia condition. No significant difference was detected between the LG- and HG-treated cells under the same hypoxia condition. From the above results, it seems that migration of ASCs was not impaired when they were exposed to the HG and hypoxia *in vitro*, an environment mimicking what happened in the ischaemic skin flap in diabetic mice.

ASCs enhanced production of VEGF and HIF1- α in diabetic flap

Given that accumulative evidence demonstrated that diabetes attenuates VEGF production and diminished levels of HIF1- α have

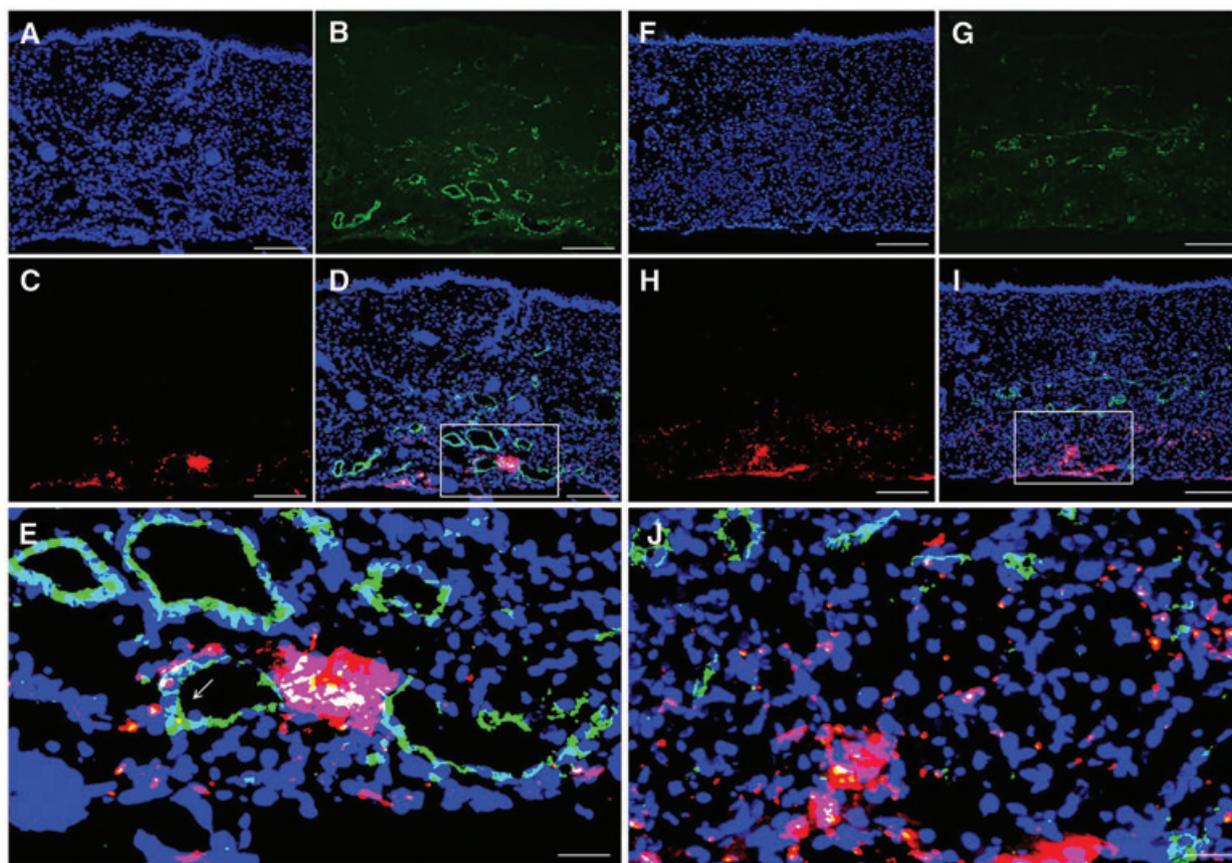


Fig. 3 Migration and differentiation of Dil-labelled ASCs in the flap of diabetic mice by post-operative day 7 (**A–E**) and day 14 (**F–J**). (**A, F**) Nuclei (blue) were stained with Hoechst. (**B, G**) Endothelial cells (green) were immunostained with an anti-CD31 antibody. (**C, H**) ASCs labelled by Dil (red) appeared in the flap tissue. (**D**) Merged images of **A–C**. (**I**) Merged image of **F–H**. (**E, J**) Localized magnification of **D** and **I**. One of the engrafted Dil-labelled ASCs was found incorporated into the blood vessel wall (yellow) indicated by a white arrow in **E**. Scale bars: 100 μm (**A–I**) and 25 μm (**E, J**).

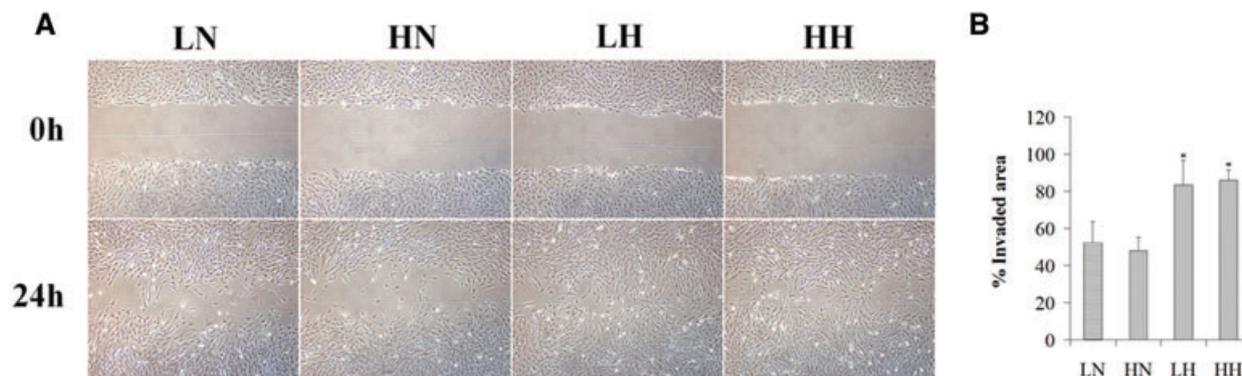


Fig. 4 Migration of ASCs in low glucose (LG) or high glucose (HG) and exposed to normoxia or hypoxia. (**A**) Phase contrast photographs of the cultures taken at 0 hr (immediately after scratching) and at the 24 hrs after scratching, showing the wound closure by cells in a random migration manner. (**B**) Quantification of the wounded area invaded at 24 hrs after scratching was represented as the percentage of wounded area at 24 hrs over that of 0 hr. $n = 5$, $P < 0.05$ versus M and B groups, respectively. LN: low glucose and normoxia; HN: high glucose and normoxia; LH: low glucose and hypoxia; HH: high glucose and hypoxia.

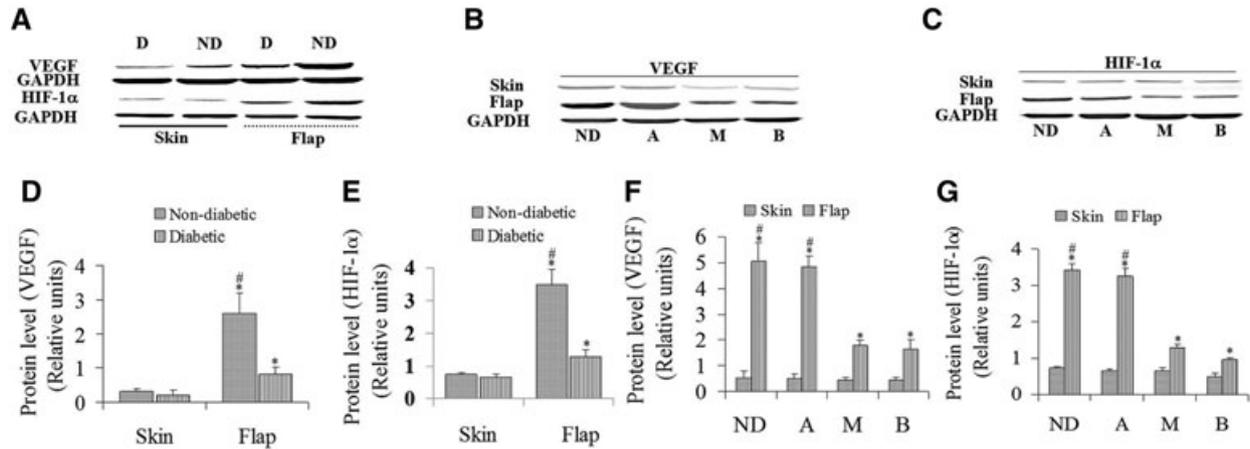


Fig. 5 ASCs augment HIF-1 α and VEGF expression in the flap tissue of diabetic mice. HIF-1 α and VEGF levels in ischaemic skin segments of non-diabetic and diabetic mice were measured 72 hrs after surgery. **(A)** Flaps from diabetic mice produced less VEGF and HIF-1 α than non-diabetic ones. **(B)** VEGF protein expression was significantly augmented in ischaemic skin segments of diabetic mice in ASCs group. **(C)** HIF-1 α protein expression was significantly augmented in ischaemic skin segments of STZ-diabetic mice in ASCs group. **(D, E)** Western blot quantitation of **(A)**. $n = 4$, * $P < 0.05$ versus skin. $n = 4$, # $P < 0.05$ versus diabetic, respectively. **(F, G)** Western blot quantitation of **(B)** and **(C)**, respectively. $n = 4$, * $P < 0.05$ versus skin. $n = 4$, # $P < 0.05$ versus M and B groups, respectively. A: ASCs-treated group; M: medium-treated group; B: blank; D: diabetic; ND: non-diabetic.

been attributed to decreased VEGF in diabetic wound, expression of VEGF and HIF1- α in diabetic flap was evaluated in this study (Fig. 5A, D and E). Although no significant difference in the expression of VEGF was detectable between normal and diabetic skin, its expression was up-regulated in the skin flap at 24 hrs after elevation (Fig. 5A and D). Similar phenomena were also observed on the expression of HIF1- α (Fig. 5A and E). Moreover, expression of VEGF and HIF1- α in the diabetic flap was significantly down-regulated when compared to that in non-diabetic flap at 24-hr post-injury.

The earlier results indicate that ASCs did not participate directly in angiogenesis, and diabetic flap had a diminished response to ischaemia. We therefore asked whether ASCs could enhance angiogenesis through releasing angiogenic cytokines. To answer this, production of VEGF and HIF1- α in ASCs-treated, medium-treated and non-treatment diabetic flap was detected by Western blot analysis (Fig. 5B, C, F and G). Compared with that in normal non-diabetic flap, expression of VEGF was dramatically decreased in medium-treated and non-treated diabetic flap, respectively ($P < 0.05$). With transplantation of ASCs, VEGF production recovered to a high-level close to that in non-diabetic normal flap, and no significant difference existed between the two groups ($P > 0.05$). In consistence with this result, introduction of ASCs into diabetic flap also rescued HIF1- α expression to 94.7% of what was detected in non-diabetic normal flap. The HIF1- α expression in ASCs group was significant higher than that either in medium-treated or non-treated flaps ($P < 0.05$), respectively.

ASCs cultured in HG maintained angiogenic response to hypoxia

On the basis of the earlier observation, we proceeded to evaluate whether cellular exposure to an HG environment would up-regulate expression of VEGF and HIF1- α in response to hypoxia stimulation (Fig. 6). Cells growing in HG medium for 1–3 days were subsequently subjected to hypoxia condition (1%) for 24 hrs, respectively, and their corresponding VEGF and HIF1- α protein levels were measured. Under normoxia circumstance (21%), cells cultured in HG medium for 1–3 days expressed similar VEGF levels to cells cultured in LG medium, respectively (Fig. 6A). When subjected to hypoxia, cells cultured in either LG or HG media for different time points exhibited a significant increase in VEGF production compared to their respective ones under normoxia ($P < 0.05$) (Fig. 6A). It is noticeable that the VEGF level in cells grown in HG for 1 day reached a high extend (3.7-fold of the corresponding one in normoxia condition), and no significant difference in VEGF expression was observed between the cells grown in HG and in LG under that same hypoxia stimulation ($P < 0.05$) (Fig. 6A). In addition, hypoxia stimulated 1.6- and 1.5-fold enhancement in VEGF expression in cells cultured in HG for 2 and 3 days compared to their respective ones at normoxia condition, respectively (Fig. 6A). However, under the same hypoxia condition, the elevated VEGF levels in cells after 2 and 3 days in HG reached only 56% and 51% of that in cells incubated in HG for 1 day, respectively (Fig. 6A).

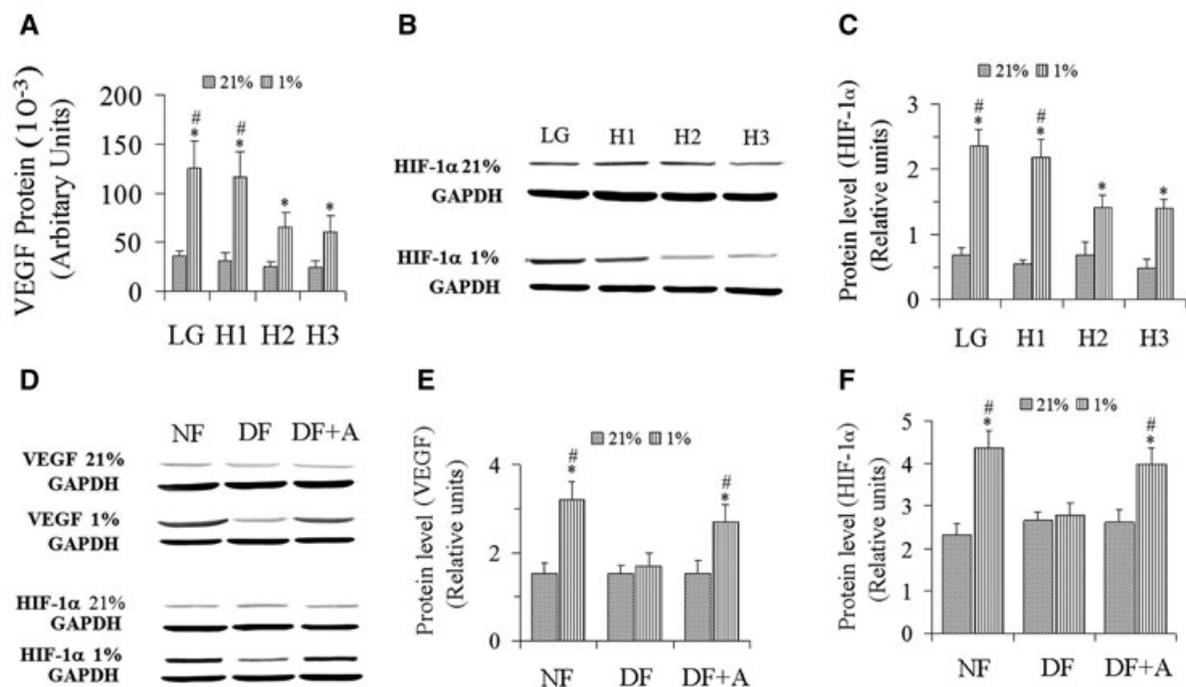


Fig. 6 Hypoxia up-regulated the protein expression of HIF-1 α and VEGF of the ASCs cultured in low or high glucose concentrations. Co-cultured ASCs promoted VEGF and HIF-1 α expression in diabetic dermal fibroblasts. **(A)** Hypoxia significantly up-regulated VEGF proteins in LG or HG medium. **(B)** HIF-1 α expression in ASCs group was augmented in LG or HG exposed hypoxia. **(C)** Western blot quantitation of **(B)**. $n = 4$, $*P < 0.05$ versus normoxia. $n = 4$, $^{\#}P < 0.05$ versus H2 and H3 groups, respectively. **(D–F)** Transwell coculture of diabetic dermal fibroblasts (DF) with ASCs. **(D)** VEGF and HIF-1 α expression in diabetic dermal fibroblasts, ASCs promoted VEGF and HIF-1 α expression in diabetic dermal fibroblasts. **(E, F)** Western blot quantitation of VEGF and HIF-1 μ . **(D)**. $n = 4$, $*P < 0.05$ versus normoxia. $n = 4$, $^{\#}P < 0.05$ versus DF group, respectively. NF: dermal fibroblasts isolated from non-diabetic mice; DF: dermal fibroblasts isolated from diabetic mice; DF+A: diabetic dermal fibroblasts that coculture of with ASCs; LG: low glucose; H1: high glucose for 1 day; H2: high glucose for 2 days; H3: high glucose for 3 days.

Interestingly, a similar pattern of HIF-1 α production was also observed when cells were exposed to hypoxia condition (Fig. 6B and C). Under normoxia condition, cells cultured in HG for 1–3 days, respectively, all elicited a comparable level of HIF-1 α protein production to cells in LG. After being subjected to hypoxia for 24 hrs, cells which had been incubated in HG for 1 day before, increased their HIF-1 α production to a similar level as cells grown in LG ($P > 0.05$). Compared with their respective ones at normoxia condition, a 2.47-fold increase in the HIF-1 α production was observed for cells grown in LG when exposed to hypoxia for 24 hrs, whereas a 3.03-fold increase was obtained for cells grown in HG for 1 day. However, cells cultured in HG for 2 and 3 days increased HIF-1 α expression by only 1.08- and 1.95-fold compared to their respective ones at normoxia condition.

ASCs promoted VEGF and HIF-1 α expression in diabetic dermal fibroblasts

DFs play a critical role in cutaneous wound healing, whereas dysfunction of fibroblasts in VEGF production and in its response to

hypoxia has been indicated in impaired wound healing in diabetics. Hence, to investigate whether transplantation of ASCs would rescue the impaired angiogenic effect of diabetic fibroblasts, expression of VEGF and HIF-1 α was detected in fibroblasts cocultured with ASCs in a transwell system (Fig. 6D–F). In consistence with previous reports, DFs from diabetic mice exhibited an impaired response to hypoxia characterized by their reduced expression of VEGF, reaching only 53% of that released by non-diabetic fibroblasts. However, when cocultured with ASCs in hypoxia condition, expression of VEGF in diabetic fibroblasts was dramatically improved to 84% of that produced by non-diabetic fibroblasts ($p < 0.05$) (Fig. 6D and E).

Similar trend in the HIF-1 α expression was also observed as that of VEGF production (Fig. 6D and F). Under normoxia condition, expression of HIF-1 α in normal fibroblasts, diabetic and ASCs-treated diabetic fibroblasts showed a comparable level ($P > 0.05$). When exposed to hypoxia, diabetic fibroblasts failed to improve their HIF-1 α expression as did by non-diabetic fibroblasts. However, when cocultured with ASCs, expression of HIF-1 α in diabetic fibroblast was remarkably enhanced in response to hypoxia, reaching a similar level to that in non-diabetic fibroblasts ($P > 0.05$).

Discussion

The high tendency of necrosis discounted greatly the outcome of random skin flap in dealing with chronic wounds in patients suffered from DM. It was reported that the survival rate of random flap in diabetic individuals was 45% in average, significantly lower than that of non-diabetic flaps [25]. Hyperglycaemia and hypoxia are suggested to play essential pathophysiological roles in increased flap necrosis in diabetes, which lead to defective neoangiogenesis in tissues responding to low oxygen tension [4, 26]. Thus, the strategy of improving viability of ischaemic diabetic flap is to augment blood perfusion *via* stimulating neovascularization tropically by means of growth factors introduction [27], angiogenic gene transfection [28] as well as stem cell delivery [29].

In this study, we demonstrated that topical application of ASCs improved viability of random skin flap in STZ-induced diabetic mice. By injection of ASCs locally, viability of skin flap was markedly increased from 47.0% in the sham control to 83.2% in ASCs-treated group. Meanwhile, the increased viability in diabetic flap was concurrent with improvement of blood perfusion as determined by laser Doppler flowmetry detection. In addition, by CD31⁺ cell calculating, it was found that capillary density was greatly enhanced in ASCs-treated flap in comparison to the control groups. In the aggregate, these data demonstrated that improved viability of diabetic skin flap by local transplantation of ASCs was a result of stimulated neovascularization.

As reported by previous studies [29] that bone marrow mesenchymal stem cells rescued the survival of skin flap by incorporating into blood vessels *via* differentiating into endothelial lineage, we therefore investigated whether ASCs took a similar endothelial pathway after they were injected *in vivo*. Opposed to our speculation, at day 7 post-implantation, no Dil-labelled ASCs could be identified within capillary vessels and transplanted cells were observed in an aggregated state in subcutaneous tissues. Even after 14 days of operation, there was no detectable fluorescent labelling cells incorporated into capillary vessels. Migration to approach capillaries is the first step for the implanted ASCs to take part in vessel structure. Lerman *et al.* [30] reported that migration of diabetic DFs was markedly impaired with exposure to hyperglycaemia. However, ASCs exposed to hyperglycaemia in combination with hypoxia culture conditions exhibited non-reduced migration ability as compared with cells cultured under normal circumstances (regular glucose levels and normoxia). Taken together, the increased angiogenesis in ASCs-treated diabetic tissue was not likely a consequence of endothelial differentiation of ASCs.

In the past few years, the use of MSCs for therapeutic purposes to improve viability of ischaemic skin flaps has been well-documented [7, 29]. It is believed that, in addition to their direct participation in vascular structures [7, 29], secretion of angiogenic cytokines [7] such as VEGF, is one of the critical issues responsible for enhanced neovascularization in MSCs-treated ischaemic skin flap [31]. On the other hand, it was reported that hyperglycaemia attenuated VEGF production [2, 4], and diminished levels of VEGF were observed in wounds of diabetic animal models [2, 25].

On the basis of this, we determined to investigate expression of VEGF in diabetic flap before and after ASCs treatment. It was found that exposure to hypoxia induced a significant increase in VEGF production by ASCs cultured at HG levels. These *in vitro* results may partially explain why an elevated level of VEGF was detected in ASCs-treated diabetic flap. In addition to VEGF, some other cytokines including bFGF, insulin-like growth factor (IGF) and stroma-derived factor (SDF) have been reported to be involved in promoting angiogenesis by locally delivery of mesenchymal stem cells [32]. Recently, Rasmussen *et al.* [33] demonstrated that prolonged culture at reduced oxygen level is optimal for ASCs to secrete pro-angiogenic factors including not only VEGF, but also IGF and chemokine (CXC motif) ligand 12 (CXCL12). These results support the theory that the angiogenesis evoked by MSC therapy is a complex interplay between several paracrine factors.

It is noteworthy that resident immunologic cells including mast cell, NK cell and eosinophils may lead to graft-*versus*-host disease (GVHD) with transplantation of xeno-ASCs in T cell deficient nu/nu mice in this study. It has been reported that circulating levels of VEGF was increased in GVHD mice model [34]. Moreover, many skin biopsies of GVHD were found to be associated with an increase of small vessels [35]. Thus, choosing other diabetic models such as db/db mice as a control is recommended in our future works. In consistence with our study, it was observed in a series of studies that levels of angiogenic growth factors including VEGF, bFGF and SDF-1, were decreased in diabetic skin flap [4, 5, 30]. However, the underlying regulatory mechanism accounting for such a reduction remains to be defined. As a transcription factor that mediates adaptive responses to hypoxia, hypoxia-inducible factor (HIF-1 α) has been proved to be a master regulator of the production of angiogenic factors in ischaemic tissues [4, 21, 36]. HIF-1 is a heterodimer composed of O₂ responsive HIF-1 α and constitutively expressed HIF-1 β subunits [37]. Diminished expression of HIF-1 α was indicated in impaired wound healing in diabetic mice [4]. In this study, it was also shown that expression of HIF-1 α was greatly reduced in diabetic flap. By administration of ASCs, HIF-1 α production in diabetic flap recovered to a similar level to that in non-diabetic one, which was in concurrence with improved viability of skin flap. Furthermore, our *in vitro* investigations demonstrated that ASCs exposed chronically to HG exhibited a hypoxia responsive reaction as determined by elevated expression of HIF-1 α . Taken together, the earlier results indicated that ASCs fulfilled their angiogenic stimulative function *via* improving expression of HIF-1 α , which consequently led to the up-regulation of VEGF expression. As reported by Thangarajah *et al.* [4], decreased activity of HIF-1 α in diabetes was specifically caused by impaired HIF-1 α binding to the coactivator p300, which was modified by HG-induced production of methylglyoxal. Thus, the attenuated association of p300 with HIF-1 α led to decreased angiogenic gene expression mediated by HIF-1 α transactivation. Thus, further work should be performed to explore molecular mechanism responsible for improved expression of HIF-1 α in ASCs exposed to hyperglycaemia.

DFs represent a central regulator in ECM deposition, remodeling and cell-cell communication in cutaneous wound repair.

Lerman *et al.* [30] observed that fibroblasts from skin of diabetic db/db mice exhibited impaired migration, VEGF production and response to hypoxia, a cellular dysfunction developed concurrently with onset of hyperglycaemia. However, no reports, up to now, have investigated the role of fibroblasts in neovascularization of diabetic ischaemic flap. In this study, we found that diabetic fibroblasts failed to up-regulate the endogenous expression of both VEGF and HIF-1 α in response to hypoxia as those in non-diabetic fibroblasts. However, when cocultured with ASCs under hypoxia condition, expression of VEGF and HIF-1 α in diabetic fibroblasts was rescued to almost normal levels. Thus, ASCs exerted their angiogenic role through not only in an autocrine way, but also in a paracrine pattern, by which cocultured diabetic fibroblasts were restored in VEGF and HIF-1 α expression in hypoxia condition.

Conclusions

Briefly, our studies demonstrated that topical application of ASCs could improve viability of ischaemic random skin flap by enhancing neovascularization in STZ-induced diabetic mice. It was found that activation of HIF-1 α triggered the angiogenic cascade, which led to increased expression of VEGF in ASCs exposed to HG and hypoxia environment. Through the investigation on the interaction

between ASCs and fibroblasts in a coculture system, it was shown that ASCs restored HIF-1 α -mediated VEGF expression in diabetic fibroblasts in response to hypoxia in a paracrine manner. Hence, local administration of ASCs may serve as an alternative approach in treating necrotic random skin flap in diabetic individuals.

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Conflict of interest

None of the authors has financial interests including products, devices, or drugs associated with this article. There are no commercial associations that might pose or create a conflict of interest with information presented in this article such as consultancies, stock ownership or patent licensing arrangements.

References

- Vija L, Farge D, Gautier JF, *et al.* Mesenchymal stem cells: stem cell therapy perspectives for type 1 diabetes. *Diabetes Metab.* 2009; 35: 85–93.
- Brem H, Tomic-Canic M. Cellular and molecular basis of wound healing in diabetes. *J Clin Invest.* 2007; 117: 1219–22.
- Zhang T, Gong W, Li Z, *et al.* Efficacy of hyperbaric oxygen on survival of random pattern skin flap in diabetic rats. *Undersea Hyperb Med.* 2007; 34: 335–9.
- Thangarajah H, Yao D, Chang EI, *et al.* The molecular basis for impaired hypoxia-induced VEGF expression in diabetic tissues. *Proc Natl Acad Sci U S A.* 2009; 106: 13505–10.
- Ceradini DJ, Yao D, Grogan RH, *et al.* Decreasing intracellular superoxide corrects defective ischemia-induced new vessel formation in diabetic mice. *J Biol Chem.* 2008; 283: 10930–8.
- Capla JM, Grogan RH, Callaghan MJ, *et al.* Diabetes impairs endothelial progenitor cell-mediated blood vessel formation in response to hypoxia. *Plast Reconstr Surg.* 2007; 119: 59–70.
- Lu F, Mizuno H, Uysal CA, *et al.* Improved viability of random pattern skin flaps through the use of adipose-derived stem cells. *Plast Reconstr Surg.* 2008; 121: 50–8.
- Lee EY, Xia Y, Kim WS, *et al.* Hypoxia-enhanced wound-healing function of adipose-derived stem cells: increase in stem cell proliferation and up-regulation of VEGF and bFGF. *Wound Repair Regen.* 2009; 17: 540–7.
- Rehman J, Traktuev D, Li J, *et al.* Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation.* 2004; 109: 1292–8.
- Tapp H, Hanley EN Jr. Adipose-derived stem cells: characterization and current application in orthopaedic tissue repair. *Exp Biol Med (Maywood).* 2009; 234: 1–9.
- Zuk PA, Zhu M, Ashjian P, *et al.* Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell.* 2002; 13: 4279–95.
- Kim WS, Park BS, Sung JH, *et al.* Wound healing effect of adipose-derived stem cells: a critical role of secretory factors on human dermal fibroblasts. *J Dermatol Sci.* 2007; 48:15–24.
- Kim WS, Park BS, Park SH, *et al.* Antiwrinkle effect of adipose-derived stem cell: activation of dermal fibroblast by secretory factors. *J Dermatol Sci.* 2009; 53: 96–102.
- Chen L, Tredget EE, Wu PY, *et al.* Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS One.* 2008; 3: e1886.
- Altman AM, Matthias N, Yan Y, *et al.* Dermal matrix as a carrier for *in vivo* delivery of human adipose-derived stem cells. *Biomaterials.* 2008; 29: 1431–42.
- Amos PJ, Kapur SK, Stapor PC, *et al.* Human adipose-derived stromal cells accelerate diabetic wound healing: impact of cell formulation and delivery. *Tissue Eng Part A.* 2010; 16: 1595–606.
- Kelly BD, Hackett SF, Hirota K, *et al.* Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue

- by a constitutively active form of hypoxia-inducible factor 1. *Circ Res.* 2003; 93: 1074–81.
18. **Elson DA, Ryan HE, Snow JW, et al.** Coordinate up-regulation of hypoxia inducible factor (HIF)-1 α and HIF-1 target genes during multi-stage epidermal carcinogenesis and wound healing. *Cancer Res.* 2000; 60: 6189–95.
 19. **Li W, Li Y, Guan S, et al.** Extracellular heat shock protein-90 α : linking hypoxia to skin cell motility and wound healing. *EMBO J.* 2007; 26: 1221–33.
 20. **Ceradini DJ, Kulkarni AR, Callaghan MJ, et al.** Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nat Med.* 2004; 10: 858–64.
 21. **Botusan IR, Sunkari VG, Savu O, et al.** Stabilization of HIF-1 α is critical to improve wound healing in diabetic mice. *Proc Natl Acad Sci U S A.* 2008; 105: 19426–31.
 22. **Cui L, Liu B, Liu G, et al.** Repair of cranial bone defects with adipose derived stem cells and coral scaffold in a canine model. *Biomaterials.* 2007; 28: 5477–86.
 23. **Liu G, Zhou H, Li Y, et al.** Evaluation of the viability and osteogenic differentiation of cryopreserved human adipose-derived stem cells. *Cryobiology.* 2008; 57: 18–24.
 24. **Moreno-Bueno G, Peinado H, Molina P, et al.** The morphological and molecular features of the epithelial-to-mesenchymal transition. *Nat Protoc.* 2009; 4: 1591–613.
 25. **Emekli U, Tuncer S, Kabakas F, et al.** The effect of short- versus long-term administration of alpha tocopherol on the survival of random flaps in experimental diabetes mellitus. *J Diabetes Complications.* 2004; 18: 249–57.
 26. **Aronson D.** Hyperglycemia and the pathobiology of diabetic complications. *Adv Cardiol.* 2008; 45: 1–16.
 27. **Zhang F, Waller W, Lineaweaver WC.** Growth factors and flap survival. *Microsurgery.* 2004; 24: 162–7.
 28. **Zheng Y, Yi C, Xia W, et al.** Mesenchymal stem cells transduced by vascular endothelial growth factor gene for ischemic random skin flaps. *Plast Reconstr Surg.* 2008; 121: 59–69.
 29. **Simman R, Craft C, McKinney B.** Improved survival of ischemic random skin flaps through the use of bone marrow nonhematopoietic stem cells and angiogenic growth factors. *Ann Plast Surg.* 2005; 54: 546–52.
 30. **Lerman OZ, Galiano RD, Armour M, et al.** Cellular dysfunction in the diabetic fibroblast: impairment in migration, vascular endothelial growth factor production, and response to hypoxia. *Am J Pathol.* 2003; 162: 303–12.
 31. **Kryger Z, Zhang F, Dogan T, et al.** The effects of VEGF on survival of a random flap in the rat: examination of various routes of administration. *Br J Plast Surg.* 2000; 53: 234–9.
 32. **Hocking AM, Gibran NS.** Mesenchymal stem cells: paracrine signaling and differentiation during cutaneous wound repair. *Exp Cell Res.* 2010; 316: 2213–9.
 33. **Rasmussen JG, Frobert O, Pilgaard L, et al.** Prolonged hypoxic culture and trypsinization increase the pro-angiogenic potential of human adipose tissue-derived stem cells. *Cytotherapy.* 2010; 13: 318–28.
 34. **Detmar M, Brown LF, Schon MP, et al.** Increased microvascular density and enhanced leukocyte rolling and adhesion in the skin of VEGF transgenic mice. *J Invest Dermatol.* 1998; 111: 1–6.
 35. **Fleming JN, Shulman HM, Nash RA, et al.** Cutaneous chronic graft-versus-host disease does not have the abnormal endothelial phenotype or vascular rarefaction characteristic of systemic sclerosis. *PLoS One.* 2009; 4: e6203.
 36. **Catrina SB, Okamoto K, Pereira T, et al.** Hyperglycemia regulates hypoxia-inducible factor-1 α protein stability and function. *Diabetes.* 2004; 53: 3226–32.
 37. **Semenza GL.** Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. *Trends Mol Med.* 2001; 7: 345–50.