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Placenta mesenchymal stem cell accelerates wound healing by enhancing angiogenesis in diabetic Goto-Kakizaki (GK) rats

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ABSTRACT

Multipotent mesenchymal stem cells have recently emerged as an attractive cell type for the treatment of diabetes-associated wounds. The purpose of this study was to examine the potential biological function of human placenta-derived mesenchymal stem cells (PMSCs) in wound healing in diabetic Goto-Kakizaki (GK) rats. PMSCs were isolated from human placenta tissue and characterized by flow cytometry. A full-thickness circular excisional wound was created on the dorsum of each rat. Red fluorescent CM-DiI-labeled PMSCs were injected intradermally around the wound in the treatment group. After complete wound healing, full-thickness skin samples were taken from the wound sites for histological evaluation of the volume and density of vessels. Our data showed that the extent of wound closure was significantly enhanced in the PMSCs group compared with the no-graft controls. Microvessel density in wound bed biopsy sites was significantly higher in the PMSCs group compared with the no-graft controls. Most surprisingly, immunohistochemical studies confirmed that transplanted PMSCs localized to the wound tissue and were incorporated into recipient vasculature with improved angiogenesis. Notably, PMSCs secreted comparable amounts of proangiogenic molecules, such as VEGF, HGF, bFGF, TGF-β and IGF-1 at bioactive levels. This study demonstrated that PMSCs improved the wound healing rate in diabetic rats. It is speculated that this effect can be attributed to the PMSCs engraftment resulting in vascular regeneration via direct de novo differentiation and paracrine mechanisms. Thus, placenta-derived mesenchymal stem cells are implicated as a potential angiogenesis cell therapy for repair-resistant chronic wounds in diabetic patients.

1. Introduction

Diabetes mellitus is increasing in incidence and represents a major health problem in the twenty-first century. Indeed, the total number of diabetic patients has been projected to increase from 171 million in 2000 to 366 million in 2030 [1]. Chronic skin ulcers are one of the most serious consequences of diabetes, representing a major contributing factor to amputation in diabetics [2]. The annual incidence of foot ulcers among diabetic patients has been variously estimated at between 1% and 4.1%, and the annual incidence of amputation is in the range 0.21% and 1.37% [3]. The economic and social costs associated with chronic wounds are enormous and include hospital costs, disability, decreased productivity and loss of independence. A number of factors have been implicated in the predisposition to non-healing wounds observed in diabetes, including microvascular disease, peripheral neuropathy and impaired angiogenesis of wounds [4]. Of these factors, angiogenesis is considered to play a pivotal pathophysiological role by virtue of its requirement in successful wound repair.

Human mesenchymal stem cells (MSCs)-based therapeutic angiogenesis has emerged as a promising strategy in regenerative medicine, including in the treatment of myocardial infarction [5], limb ischemia [6] and chronic skin ulcers [7,8]. Particularly, bone marrow MSCs (BM-MSCs) [9] and endothelial progenitor cells (EPCs) [10] have been subjected to the most comprehensive translational and human studies of all stem cell approaches in wound healing. However, the use of BM-MSCs and EPCs is associated with some deficiencies. The survival and differentiation potential of BM-MSCs or progenitor cells obtained from aged patients or patients with age-related disorders are impaired, thus limiting their therapeutic efficiency [11,12]. Furthermore, autologous delivery of BM-MSCs or EPCs is inevitably associated with a delay in treatment due to time required for cell collection, isolation and propagation of

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sufficient numbers for injection [13]. Therefore, there is emerging interest in the identification of alternative cell sources of MSCs.

In the present study, a population of multipotent stem cells was isolated from human term placenta, a temporary organ with fetal contributions that is discarded postpartum. These placenta mesenchymal stem cells (PMSCs) display typical mesenchymal characteristics such as great capacity for self-renewal while maintaining their multipotent differentiation potential and expression of common MSCs surface markers similar to those expressed by BM-MSCs. We hypothesized that PMSCs administration may restore wound healing in pathological conditions. Therefore, the effect of PMSCs transplantation on the wound healing rate in diabetic Goto-Kakizaki (GK) rats was evaluated. Furthermore, the mechanisms underlying the beneficial effects of PMSCs were assessed by analysis of their capacity of differentiation into endothelial cells and secretion of proangiogenic factors.

2. Materials and methods

2.1. Patient selection and tissue processing

With consent, fresh placentas were collected from normal, full-term (38–40 weeks gestation), healthy donor mothers according to the regulations of the Independent Ethics Committee of the Shanghai Ninth People's Hospital affiliated with Shanghai JiaoTong University School of Medicine. Written informed consent was signed prior to the study. Umbilical cord blood was allowed to drain from the placentas, which were then dissected carefully. All tissues were tested to exclude HIV-infection, toxoplasmosis, cytomegalovirus and rubella virus infection. To preserve cell viability, all tissues were processed within 3 h, after they were evaluated by a certified pathologist.

2.2. Placenta-derived mesenchymal stem cell isolation

The harvested pieces of tissue were washed several times in phosphate-buffered saline (PBS) and then manually minced and enzymatically digested with 0.1% collagenase (Sigma–Aldrich, St. Louis, MO) and 10% FBS (Hyclone, South Logan, UT) in PBS solution at 37 °C for 1 h. Digested tissue was filtered twice through a cell strainer (Falcon 3078, pore size 200 mm; BD Biosciences, San Jose, CA) to eliminate undigested fragments. After centrifugation at 2000 rpm for 10 min, cells were collected, and red blood cells were lysed with red blood cell lysis buffer for 5 min at 37 °C, then centrifuged at 300 × *g* for 5 min. The supernatant was discarded, and the cell pellets were resuspended in DMEM medium with 1 g/l of glucose and 10% FBS supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 1% nonessential amino acids. Cells were cultured at 37 °C, under a 5% CO_2 atmosphere for 4 days before the culture medium was first changed and a 70–80% cellular confluence was obtained. The first passage was possible after 1 week, depending on each case. The fibroblast-like phenotype was noticed after 7 days in culture. After the adherence of the first cells, the proliferation rate increased progressively, and cell morphology was kept the same for several passages.

2.3. Identifying phenotypic markers of PMSCs by FACS

PMSCs from passage 3 were used for phenotypic marker identification by flow cytometry. The cells were detached with trypsin (0.25% with 0.1% ethylenediaminetetraacetic acid (EDTA) in Hanks' balanced salt solution (HBSS)) and suspended in cold staining buffer. Approximately 5×10^5 cells were incubated with fluorescence-conjugated antibodies for 30 min. The antibodies used were CD29 (APC-conjugated), CD90 (FITC-conjugated), CD73 (PE-conjugated), CD105 (PE-conjugated), CD49b (APC-conjugated), HLA-DR (FITC-conjugated), CD45 (PE-conjugated), and CD34 (FITC-conjugated). All of the antibodies were obtained from Becton Dickinson and Company (BD Pharmingen, San Diego, CA). At least 15,000 events were analyzed by flow cytometry (FACScan, BD Biosciences), and analyses were performed with BD FACSDiva software (version 5.0, BD Biosciences).

2.4. Differentiation of PMSCs: adipogenesis, osteogenesis, chondrogenesis

To demonstrate that the cultured cells were multipotent, differentiation into adipocytes, osteocytes and chondrocytes was performed. The PMSCs at the third passage were plated at 1×10^4 cells/well in 24-well plates, and cells were allowed to adhere to the culture surface for 24 h at 37 °C. Adipogenesis, osteogenesis, and chondrogenesis were induced by replacing the growth medium with Adipogenic, osteogenic and chondrogenic differentiation bullet kits, respectively, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The differentiation phenotype was documented using oil red O staining for adipocytes, Alcian blue staining for chondrocytes, and Alizarin red staining for osteocytes.

2.5. Endothelial differentiation of PMSCs

To initially determine whether the PMSCs could develop the characteristics of endothelial cells, they were cultured in endothelial differentiation medium containing VEGF (50 ng/ml), bFGF (50 ng/ml) and heparin (Sigma–Aldrich, St. Louis, MO) on a Matrigel-coated dish for 10 days. The medium was exchanged three times per week.

2.6. Reverse transcription and real time polymerase chain reaction

Total RNA from the PMSCs was isolated with TRIZOL. Reverse transcription of 1 μg RNA was performed with the SuperScript III Reverse Transcriptase following Invitrogen's protocol with Oligo dT primer. Quantitative PCR amplification and detection were performed according to the manufacturer's protocol on an ABI Prism 7300 sequence detection system. Cycling parameters were 95 °C for 10 s, then 40 cycles of 95 °C for 5 s, and 60 °C for 34 s. As a reference gene, the mRNA level of GAPDH was determined with the real-time PCR assay for each RNA sample and was used to correct for experimental variations. Quantifications were performed in duplicate, and the experiments were repeated independently three times. Melting curves were performed using Dissociation Curves software (Applied Biosystems) to ensure that only a single product was amplified. The following primer sequences were used: OCT4 (5′-ACATCAAAGCTCTGCAGAAAGAACT-3′), (5′-CTGAATACCTTCCCAAATAGAACCC-3′), CD44, (5′-CTGTACACCCCATCCCAGAC-3′), (5′-TGTGTCTTGGTCTCTGGTAGC-3′), SSEA-4 (5′-TGGACGGGCACAACTTCATC-3′) and (5′-GGGCAGGTTCTTGGCACTCT-3′).

2.7. Immunofluorescent staining

For immunofluorescent staining of CD31 (endothelial cell adhesion molecule 1, PECAM), vWF or VE-cadherin (CD144), cells cultured in endothelial differentiation medium for 10 days were fixed for 30 min at room temperature with methanol and permeabilized for 5 min with 0.2% Triton X-100 and 1% goat serum. Cells were then incubated with anti-CD31, anti-vWF and anti-VE-cadherin antibodies (1:100, Santa Cruz Biotechnology, CA, USA) followed by incubation with appropriate secondary antibodies. Nuclei were stained with DAPI for 10 min. Photographs were taken

with a DP70 digital camera (Olympus) and analyzed using MetaMorph software (Molecular Devices).

2.8. Determination of angiogenic factors in PMSCs media cultured in normal and hypoxic conditions

PMSCs were cultured for 72 h under hypoxic conditions (2% O_2). A growth factor-free endothelial cell basal medium-2 (EBM-2, Lonza, Switzerland) with 1% FBS was employed in this step and fresh media served as background control. After incubation, the culture supernatant was collected, sterile filtered, and stored at −80 °C until use. The hypoxic-conditioned media of the PMSCs were analyzed for the angiogenic growth factors TGF-β, IGF-1, HGF, VEGF and bFGF with a Searchlight multiplex ELISA array (Pierce Biotechnology, Thermo Fisher Scientific, Rockford, IL). Data were expressed as mean ± SEM picograms of the secreted factor per 10^6 cells at the time of harvest.

2.9. Animal studies

Animal husbandry and experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the Shanghai Experimental Animal Center of the Chinese Academy of Sciences. Diabetic GK rats (aged 8–12 weeks) were placed in individual cages and subjected to wounding. The dorsum was clipped free of hair. A full-thickness circular (8 mm diameter) excisional wound was created on the dorsum of each rat using a dermal biopsy punch. Six hours after surgery, 10^6 PMSCs stained with the cell tracking dye CM-Dil dissolved in 200 μl PBS were intradermally injected around the wound area of each rat in the treatment group. An equal volume of PBS was applied to the wounds of control rats in the same manner. Plasma glucose levels were measured post-operatively on day 0, 5, 10 and 15.

2.10. Analysis of wound closure

Digital photographs of wounds were taken at day 0, 5, 10 and 15. The wound area was measured by tracing the wound margin and calculated using an image analysis program (NIH Image). The percentage wound closure was calculated as follows: (area of original wound – area of actual wound)/area of original wound × 100. The inside edge of the splint exactly matched the edge of the wound, so that the splinted hole was used to represent the original wound size [14]. Rats were heavily anesthetized using ketamine and dorsal skin was removed using aseptic technique at day 15 after the incision. Each wound was cut in two pieces. One was placed into buffered formalin solution for histopathological examination, while the other was frozen for immunohistochemical analysis.

2.11. Immunofluorescence and immunohistochemistry

Representative tissue sections were processed for routine hematoxylin and eosin (H&E) staining. Tissue for immunohistochemical analysis was paraformaldehyde fixed, paraffin embedded and sectioned for standard immunostaining. For quantitative investigation of fibrosis, inflammation and vascular differentiation, tissues were stained with Masson's trichrome, rat anti-mouse CD45, monoclonal mouse anti-human CD31, anti-human vascular endothelial growth factor and anti-human α-SMA respectively. The extent of neovascularization was quantified by measuring capillary density using ImageJ software (NIH, Bethesda, MA, USA). The number of anti-vWF-positive capillaries was counted in six sections for each sample to calculate the average positive immunoreactivity.

2.12. Fluorescence microscopic evaluation of wound vascularity

15 days after transplantation of CM-DiI-labeled PMSCs, animals were prepared for visualizing functional vasculature in the healing wound prior to euthanasia. 500 μl of FITC-UEA-l (Sigma–Aldrich, St. Louis, Mo, USA) was injected intravenously and allowed to perfuse for 10 min. The animal was then perfused with PBS and fixed with 4% paraformaldehyde. Dorsal skin was obtained using a 10 mm biopsy punch. Multiple frozen sections (thickness, 6 μm) were prepared and examined by confocal microscopy.

2.13. Statistical analysis

All results are presented as the mean ± SEM. Statistical comparisons between two groups were performed using the Student *t*-test. Probability (P) values < 0.05 were considered statistically significant. All in vitro experiments were repeated at least in triplicate and data used for analysis.

3. Results

3.1. Characterization of PMSCs

We began our studies with the derivation and passaging of PMSCs as described under methods. To characterize the phenotypes of PMSCs, flow cytometry was performed to analyze surface markers of PMSCs. In this study, most of the PMSCs strongly expressed CD29, CD90, CD73, CD105 and CD49b but were negative for HLA-DR, CD45, and CD34. The immunophenotype of PMSCs remained unchanged for more than eight cell passages. PMSCs were also shown to give rise to adipogenic, chondrogenic and osteogenic lineages upon specific induction as confirmed, respectively, by Oil red O, Alcian blue, and Alizarin red staining (Supplementary Fig. 1).

3.2. PMSCs transplantation promotes wound closure

As shown in Fig. 1A, full-thickness incisional wounds were created in the diabetic GK rat model. Six hours later, PMSCs were transplanted around the wound. The mean wound diameter was initially 8 mm. There was no significant difference in primary wound surface area among the two groups. PMSCs-treatment (vi) resulted in a significantly smaller wound than control (ii) within 5 days of treatment (percent wound area, 64.01 ± 10.03% vs. 91.32 ± 16.98%, $P < 0.01$). This effect was maximal by day 15 (viii 17.34 ± 2.01% vs. iv 39.28 ± 4.63%, $P < 0.01$) (Fig. 1B).

The biopsies of cutaneous wound healed tissues were taken on day 15 post-operation. Samples were fixed, sectioned and stained with H&E and Masson's trichrome stain. In accordance with wound closure rates, histology revealed increased cellular infiltration and collagen deposition and thick granulation tissue in PMSCs-treated wounds (Fig. 1C). The thickness of the newly formed epidermal layer in the treatment group (Fig. 1C(iii) and Fig. 1D(iii)) was much greater than that of the no-treatment group (Fig. 1C(ii) and Fig. 1D(ii)) on day 15 after transplantation. Furthermore, the alignment of fibers in the healing skin tissue appeared more regular in the PMSCs-treatment group (Fig. 1C(iii) and Fig. 1D(iii)) compared with the no-treatment group (Fig. 1C(ii) and Fig. 1D(ii)), more similar to the normal (Fig. 1C(i) and Fig. 1D(i)). Interestingly, *Folliculuspili* and some other appendices emerged in the repairing skin tissue of the PMSCs treatment group but not in the no-treatment group.

3.3. PMSCs transplantation increases new capillary formation

Activation of angiogenesis is required to sustain newly formed granulation tissue. We therefore investigated whether local

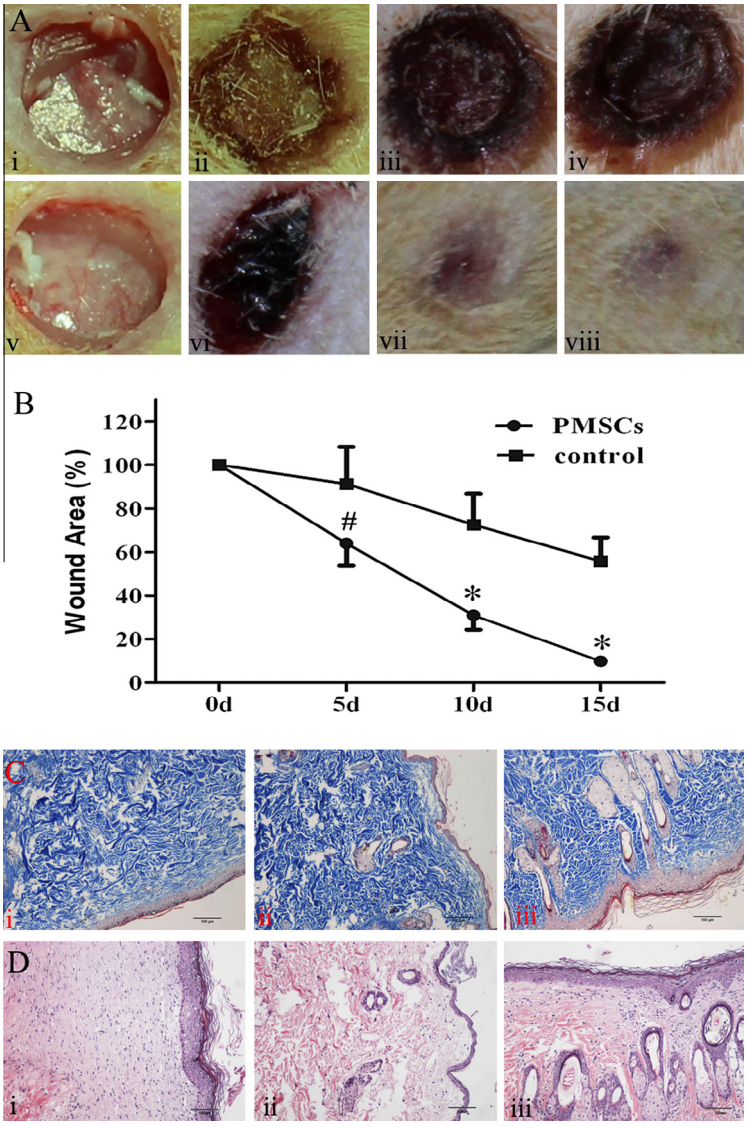

Fig. 1. Effects of PMSCs on wound closure. (A) Aspect of wounds under PBS treatments (i–iv) and PMSCs treatments (v–viii) at day 0, 5, 10 and 15. (B) Wound closure analysis. All wounds were measured using digital calipers at day 0, 5, 10 and 15 post-operation. The wound contraction rate is plotted as the percentage reduction of original wound area over time (each group, $n = 5$; $^{\#}P < 0.05$, $^{*}P < 0.01$ vs. control). (C) Masson trichrome staining showed that epidermal layer was obviously thicker in PMSCs-treated group (iii) than that of PBS-treated control (ii), more similar to the normal (i). (D) Histological changes in the cutaneous wound healed tissues following administration of PMSCs. The thickness of the newly formed epidermal layer in the PMSCs-treated group (iii) was much thicker than that in PBS-treated control (ii) on day 15 and the PMSCs-treated group exhibited a thick layer of granulation tissue with a large number of microvessels.

transplantation of PMSCs augmented neovascularization at the site of injury. Capillary density was measured by von Willebrand factor (vWF) staining of wound sections obtained at day 15 post-operation. Histologic evaluation showed that capillary density, an index of neovascularization, was markedly increased in PMSCs-transplanted mice. Quantitative analysis revealed that the capillary density in the granulation tissue was almost fourfold higher in the PMSCs-treatment group (Fig. 2A(iv–vi)) than in the no-treatment groups (Fig. 2B(i–iii)). Mean microvessel density in the PMSCs treatment group at 15 days post-operation was 16.52 ± 2.36 vessels per high-power field, whereas density in the no-treatment group at this time-point was 4.35 ± 0.73 vessels (Fig. 2B, $^{*}P < 0.01$).

To confirm whether PMSCs are directly involved in the development of vasculogenesis in the wound, PMSCs were labeled with CM-DiI for the purposes of tracking. This enabled the identification of placenta-derived cells. Previous in vivo experiments have shown that dye-labeled cells, such as fibroblasts, are still present and detectable in unwounded skin after 28 days. Incisional wounds treated with labeled PMSCs were imaged at day 15. Green fluorescent FITC-UEA-1, a marker for endothelial cells, was injected intravenously to enhance the contrast of perfused vessels and investigate the connection of the vascular networks with the rat circulatory vasculature (Fig. 2C). FITC-UEA-l binding endothelial cells in functional vessels and CM-DiI-labeled PMSCs were visualized as green and red fluorescence, respectively. The data obtained suggested successful integration of the newly formed functional endothelial cells into the local tissue vascular networks. Cord-like networks that developed from CM-DiI-labeled PMSCs were observed as early as 4 days after implantation, thus suggesting that PMSCs are directly involved in vasculogenesis in this diabetic model.

3.4. Differentiation ability of PMSCs in acute full-thickness skin wounds

Differential effects of PMSCs in acute full-thickness skin wounds were also observed. Wounds exhibited significant recovery around

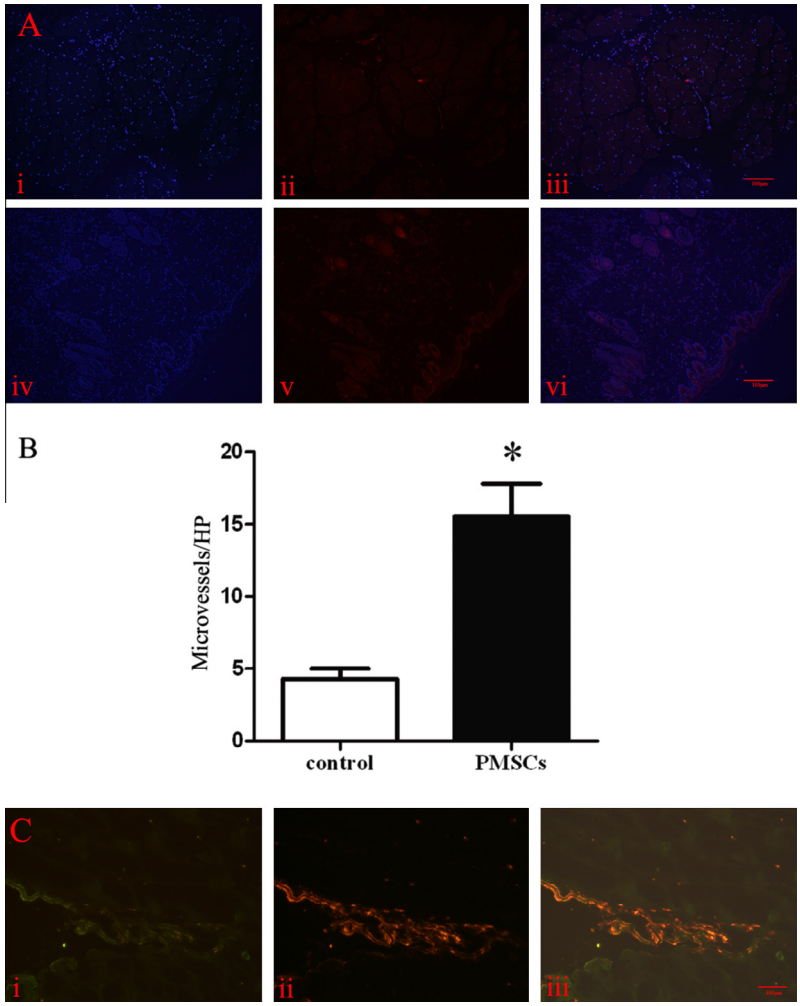

Fig. 2. PMSCs transplantation increases new capillary formation. (A) Immunostaining stained image of healed wound bed region. Representative image showing 4,6-diamidino-2-phenylindole (DAPI) stain highlighting nuclei (blue, i and iv) and stain for von Willebrand factor (red, ii and v) colocalizing with nuclear signal and outlining the luminal cross-sections of microvessels in dermal tissue biopsy obtained at 15 days post-operation. (B) Mean microvessel density at 15 days post-operation ($^*P < 0.01$). (C) Transplanted PMSCs differentiated into endothelial lineage cells and participated in the vessel network in vivo. (i) Murine capillary network stained by FITC-UEA-I (blue). (ii) CM-DiI-labeled cells were sprouting from local injection (red). (iii) Merged images of (i) and (ii) showed the incorporation of placenta stem cells into murine vascular networks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

day 15 after PMSCs-treatment. Therefore, immunostaining analysis was performed at this time-point. Anti-human CD31 staining revealed significantly different overall neovascularization in the PMSCs-treatment rats (Fig. 3A(iv–vi)).

The improvement in angiogenesis was further supported by α-SMA analysis. histological analysis showed that human α-SMA$^+$ cells were detected in PMSCs-treated tissues at day 15 (Fig. 3B(ii)). α-SMA$^+$ cells were detected in small arteries. These results demonstrated that PMSCs contributed to arteriole formation. Immunohistochemical staining showed wide distribution of VEGF produced by cells that stimulate vasculogenesis and angiogenesis, around the newly formed vessels (Fig. 3C(iv–vi)). We assayed for the recruitment of CD45+ leukocytes as an indicator for inflammation, PMSCs treatment group had significantly less inflammation (Fig. 3D(iv–vi)) than the no-treatment group (Fig. 3D(i–iii)).

3.5. PMSCs undergo in vitro differentiation into endothelial-like cells

It has also been suggested that different culture conditions may exert different impacts on transcriptome, proteome, and cellular architecture of MSCs. MSCs are able to differentiate into functional cells—not only mesoderm but also endoderm. PMSCs exhibit multilineal differentiation ability and research has shown that cells derived from fresh PMSCs can transdifferentiate into insulin-secreting cells. We postulated that PMSCs could potentially differentiate into the endothelial cell lineage. According to previous studies on the induction of MSCs into endothelial cells [9], we formulated a 10-day differentiation protocol for the conversion of endothelial cells from PMSCs.

In the process of culture, PMSCs become plastic adherent, form spindle-shaped cells (Fig. 4A), and express a panel of endothelial-specific markers. We used endothelial-specific marker antibodies against vWF, CD31, and VE-cadherin to identify cells. After PMSCs were induced for 10 days, cells were stained with fluorescence-labeled anti-vWF antibodies, anti-CD31 antibodies and anti-VE-cadherin antibodies (Fig. 4B). Undifferentiated PMSCs showed almost no specific staining for vWF, CD31, and VE-cadherin.

It has been reported that the SSEA-4, Nestin, CD44, CD29 and Oct-4 genes are expressed in several human adult stem cells. Therefore, real-time PCR was performed for the expression of SSEA-4, CD44, and Oct-4 in PMSCs. When expanded in culture, PMSCs showed a multilineal differentiation capacity and expressed the embryonic stem cell markers for SSEA-4, CD44, and Oct-4. The levels of Oct-4 and SSEA-4, which are known to be expressed in hESCs, served as a control. SSEA-4 and Oct-4 genes were expressed in the undifferentiated stage of the cells at day 0. SSEA-4 and Oct-4

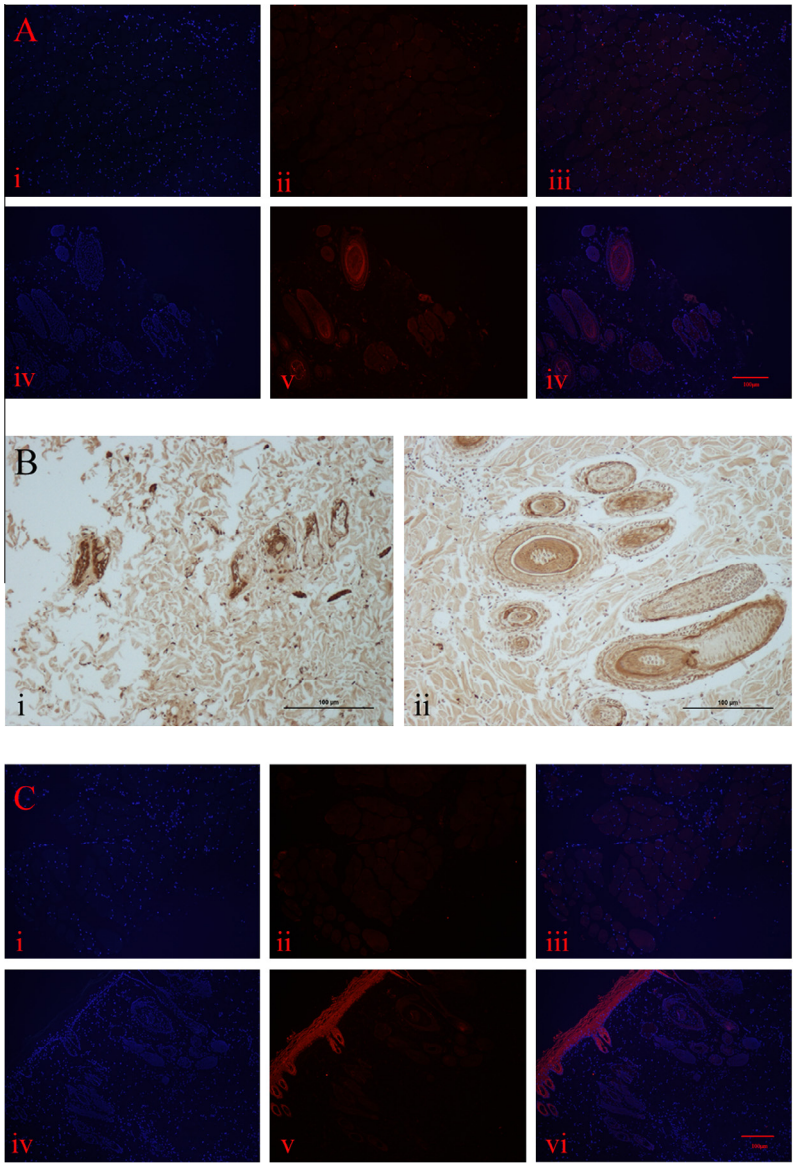

Fig. 3. Differentiation ability of PMSCs. (A) Representative images of endothelial differentiation following CD31+ staining. CD31 staining revealed that there were more capillaries were apparent in the PMSCs-treated group (iv–vi) than in PBS-treated group (i–iii). (B) Smooth muscle differentiation with α-SMA$^+$ stain. The number of α-SMA$^+$ lumen-containing vascular structures in the group receiving PMSCs (ii) was significantly greater compared with the control group (i). (C) Immunocytochemical examination revealed weakly positive expression of VEGF in the control group (i–iii) and strongly positive expression of VEGF in the PMSCs-treated group (iv–vi). (D) CD45+ staining of inflammation. The PMSCs-treated group had significantly less inflammation (iv–vi) than the control group (i–iii). (E) The 2-group comparison of CD31, α-SMA, VEGF and CD45+, staining was significantly different ($^{\#}P < 0.05$, $^{*}P < 0.01$).

decreased notably after endothelial cells formation and differentiation at day 10 (Fig. 4C).

3.6. Contribution of PMSCs paracrine factors to wound healing

MSCs are known to secrete multiple angiogenic growth factors, such as VEGF and HGF, at levels that are bioactive. Supernatants of PMSCs in 1% FBS culture medium were harvested after exposure to hypoxic conditions (2% O_2) for 72 h. VEGF, HGF, bFGF, transforming growth factor (TGF)-β and insulin like growth factor-1 (IGF-1) were detected in both groups. As shown in Fig. 4D, the amounts of VEGF, IGF-1 and bFGF were markedly higher under hypoxic conditions. In particular, a consistent and prominent augmentation was observed in the secretion of VEGF, which increased nearly fivefold.

4. Discussion

The major goal of this study was to investigate the impact of transplanted PMSCs on the wound healing process in diabetic rats. To this end, our results demonstrate for the first time that PMSCs transplantation remarkably increased the wound healing rate and improved the condition of the scar by histological evaluation as well as by surface inspection. The greater potential of PMSCs may be related to the vascular differentiation capacity of these cells, as well as paracrine mechanisms. Local intradermal injection of PMSCs had no effect on the plasma glucose level (Supplementary Table 1). Therefore, the observed effect on wound healing cannot be attributed to this parameter. Thus, our findings provide proof of concept that MSCs can be generated from human placenta with a robust differentiation potential and are suitable for use in tissue repair and engineering.

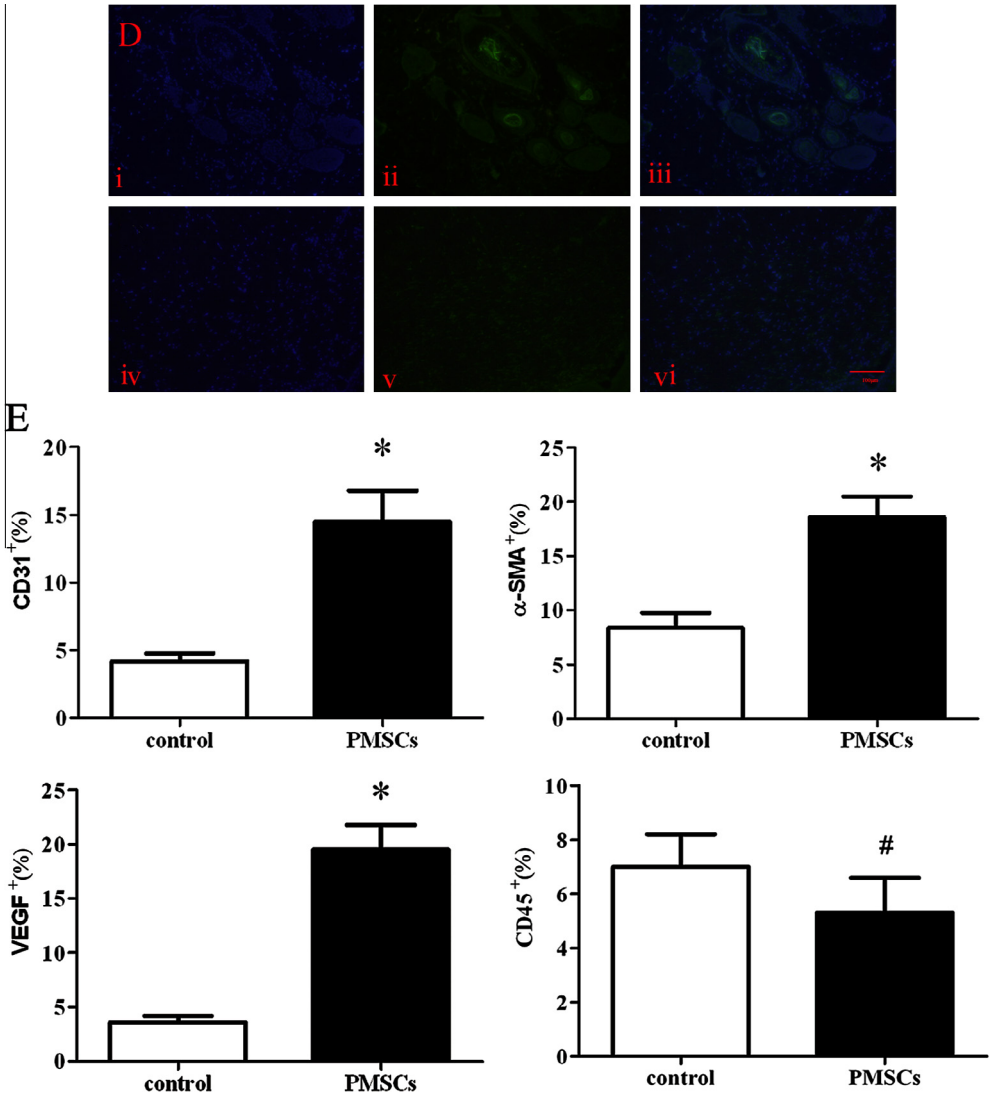

Fig. 3 (*continued*)

Multipotent MSCs, especially those derived from BM, have recently emerged as an attractive cell type for the treatment of vascular disease [15], myocardial ischemia [16] and repair-resistant chronic wounds in diabetes [17]. However, it has been estimated that MSCs represent only a minute fraction (0.01–0.001%) of the nucleated cell population in adult human bone marrow [13,18]. The limitations of these cells have increased the interest in other sources for stem cell therapy. In this study we sought to establish a protocol for the generation of a high yield of MSCs from human placenta. Compared with conventional BM stem cells, the use of PMSCs is associated with significant advantages. First, human placentas are medical waste material and are abundantly available from maternity wards, are free from ethical concerns. Second, a further advantage of the terminal placenta is that, unlike BMSCs, no invasive procedures are required to obtain MSCs. PMSCs can be easily isolated and expanded without morphological changes. Third, PMSCs contain pluripotent cells that can differentiate along multiple cell lineages. The Oct-4 and SSEA-4 expression are believed to be essential for the regulation of the differentiation state [16,19]. Our data on the differentiation capacities of PMSCs are promising and indicate the great potential of these cells for therapeutic purposes. Fourth, PMSCs treatment at the doses investigated in this study did not cause massive rejection but enhanced angiogenesis. Thus, PMSCs are implicated as a novel source for cell therapy in tissue repair and engineering.

Diminished peripheral blood flow and decreased local neovascularization are critical factors that contribute to delayed or repair-resistant wounds in diabetics [2]. The correction of impaired local angiogenesis may be a key component in developing therapeutic protocols for the treatment of chronic wounds of the lower extremities and diabetic foot ulcers [20,21]. In the present study, we demonstrated that human placenta tissue-derived stromal cells improve the wound healing rate in diabetic rats through increasing angiogenesis and collagen accumulation in the wound tissue. It is now well established that an essential part of normal healing for full-thickness cutaneous wounds is the formation of new blood vessels within the provisional wound matrix, which is referred to as granulation tissue. In an in vitro study in which PMSCs were cultured in endothelial cell conditioned medium, cells acquired endothelial-like characteristics and expressed endothelial markers CD31, vWF and VE-cadherin [19,22]. The capacity of PMSCs to differentiate into endothelial cells indicated the potential of these cells to participate in angiogenesis. Similarly, histologic evaluation of sections retrieved from the site of wound repair in mice sacrificed at day 15 showed that capillary density, an index of neovascularization, was markedly increased in PMSCs-transplanted wounds.

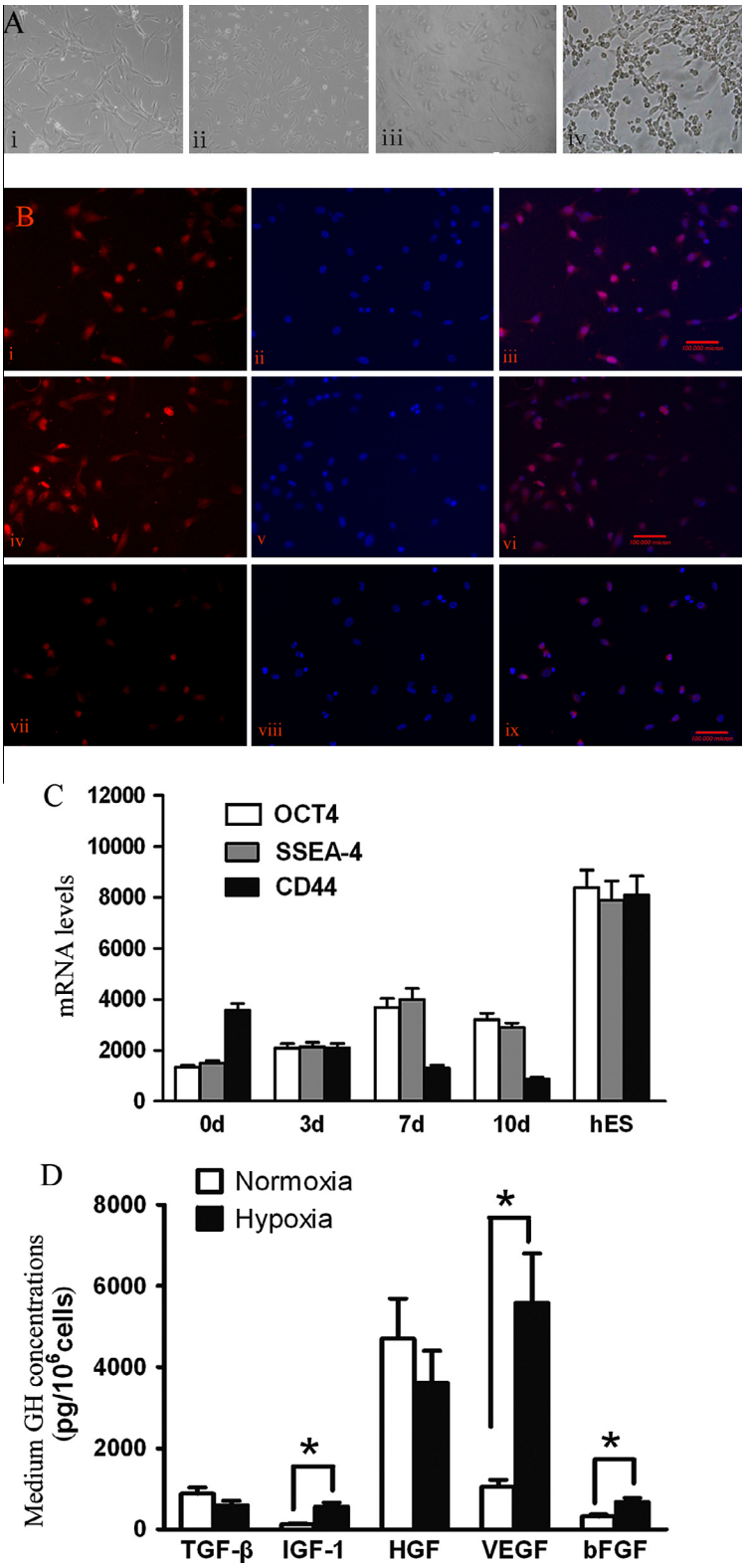

Fig. 4. PMSCs undergo in vitro differentiation into endothelial cells. (A) Morphological changes and characterization of endothelial cell differentiation from PMSCs during the 10-day procedure. (i) PMSCs were plated on Matrigel-coated culture dishes on the first day. (ii) Four days after plating, adherent cells with shorter spindle appeared. (iii) Spindle-shaped cells were formed in 7 days. (iv) Numerous spindle-shaped cells appeared after 10 days in culture. (B) Expression of endothelial cell markers differentiated cells. Cells were differentiated and stained with antibodies against CD31 (i–iii, red), vWF (iv–vi, red), VE-cadherin (vii–ix, red), and DAPI nuclear staining (blue) on day 10. (C) Gene expression during PMSCs differentiation. SYBR Green-based qRT-PCR was performed for ESC marker genes like OCT-4, SSEA-4, and CD44 at day 0, day 3, day 7 and day 10 (0d, 3d, 7d and 10d) during the differentiation process. Relative levels of gene expression were normalized to the GAPDH mRNA level and are presented here as the mean ± SEM fold increase over detectable levels. (D) Secretion of TGF-β, HGF, VEGF, bFGF and IGF-1 by PMSCs cultured in normal or hypoxic conditions over 72 h was measured by ELISA and is presented as mean ± SEM picograms of secreted factor normalized to 10^6 cells at the time of harvest. Growth factor production in normal and hypoxic conditions was compared using a paired *t*-test (*$P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

It is well known that peripheral vascular disease (macroangiopathy), along with diabetic neuropathy, plays a major role in diabetic foot ulceration. Restoring blood flow to the site of injured tissue is a prerequisite for mounting a successful repair response [23]. Interestingly, we showed that transplanted PMSCs were incorporated into recipient vasculature and improved angiogenesis with an associated increase in capillary density and blood perfusion. New blood vessels within the wound supply oxygen and nutrients to support the cellular proliferation involved in tissue restoration [24]. It has been reported that MSCs from different tissues (adipose tissue [25], bone marrow [26], umbilical cord [27] and skin [28] can differentiate into vascular endothelial cells. However, direct in vivo evidence for the contribution of MSC-derived endothelial cells to new vessel formation has not yet been reported. We evaluated the capacity of PMSCs labeled with red fluorescent CM-DiI to form functional blood vessels in vivo. Cord-like networks that developed from CM-DiI-labeled PMSCs were observed as early as day 4 after implantation. To confirm the connection of the engineered vascular network with the rat circulatory system, blue fluorescent FITC-labeled-UEA-I were injected intravenously to enhance the contrast of perfused vessels. Incorporation of some PMSCs within the recipient vasculature was observed. Thus, our study demonstrates that PMSC transplantation may be used to successfully promote neovascularization of wound repair.

It has also been suggested that MSCs secrete a broad spectrum of angiogenic or anti-apoptotic cytokines that play a role in the process of tissue repair [29]. Several studies have shown that adult MSCs secrete a variety of cytokines [30,31] BM-MSC-conditioned medium has been shown to contain the majority of the 79 human cytokines, including growth factors and chemokines, in antibody-based protein array analysis [32]. Optimum healing of a wound requires a well-orchestrated integration of many molecular events mediated by cytokines [8]. VEGF, IGF-1, bFGF and TGF-β are among the most potent angiogenic cytokines involved in ischemia angiogenesis. For example, ADSC-conditioned medium improved perfusion in hind-limb ischemia induced by femoral artery ligation, which appears to be mainly achieved by the capacity of these cells to secrete paracrine factors [33]. In the present study we found that PMSCs after cultured under to hypoxic conditions (2% O_2) released a comparable amount of proangiogenic molecules, such as VEGF, HGF, bFGF, TGF-β and IGF-1 at levels that are bioactive. Of the differentially expressed growth factors, VEGF is particularly intriguing as the expression of VEGF in PMSCs is extremely high and this factor has recently been shown to play a critical role in the regeneration of various tissues [34]. Thus, it can be speculated that VEGF, along with other differentially expressed cytokines, is involved in cutaneous regeneration.

In summary, our data indicate that PMSCs represent a promising cell source for the treatment of repair-resistant diabetic wounds. PMSCs improved the wound healing rate in diabetic rats. The great potential of PMSCs may be attributed to their engraftment after transplantation resulting in induction of vascular regeneration via direct de novo differentiation and paracrine mechanisms. Further investigations are required to elucidate the exact mechanism by which PMSCs interact to confer benefit during the process of wound healing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.088.

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