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Original Article

Hypoxia enhances the healing effect of placental-derived mesenchymal stem cells on scald wound by promoting secretion of insulin-like growth factor-1

Lili Du[1], Runxiao Lv[2,3], Xiaoyi Yang[4], Haiying Ma[1], Yanqiu Yu[1]

[1]Department of Pathophysiology, College of Basic Medical Science, China Medical University, Shenyang 110122, People's Republic of China; [2]Graduate Management Unit, The Second Military Medical University, Shanghai 200433, People's Republic of China; [3]Department of Orthopedics, Changhai Hospital, The Second Military Medical University, Shanghai 200433, People's Republic of China; [4]Seven-Year System, Department of Clinical Medicine, China Medical University, Shenyang 110122, People's Republic of China

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Abstract: Accumulating evidences have indicated that mesenchymal stem cells (MSCs) are able to promote scald wound healing through paracrines. This study aims to investigate whether hypoxia can enhance the healing effect of human placenta-derived MSCs (pMSCs) on scald wound through paracrines and to explore the underlying mechanism. The expression of hypoxia-inducible factor 1 and insulin like growth factor-1 (IGF-1) was significantly increased in pMSCs under hypoxia as compared to normoxia. Fibroblasts and human umbilical vein endothelial cells (HUVECs) grew faster when cultured in pMSCs-derived hypoxic conditioned medium (hypoCM) compared with the counterparts cultured in pMSCs-derived normoxic conditioned medium (norCM), whereas cell proliferation was significantly inhibited when anti-IGF-1 antibody (IGF-1Ab) was added. Wound scratch assay showed that hypoCM promoted migration of fibroblasts, HUVECs and macrophages. Interestingly, IGF-1Ab attenuated hypoCM-enhanced mobility of fibroblasts and HUVECs, but had no effect on macrophage migration. Compared with norCM, hypoCM promoted tube formation of HUVECs *in vitro*, which was dampened by IGF-1Ab. In the *in vivo* scald wound healing model, the wound healed faster after treatment with hypoCM than that treated with norCM, and the healing effect of hypoCM was reduced in the presence of IGF-1Ab. Real-time PCR and immunohistochemistry revealed that hypoCM significantly dowregulated the expression of type I and type III collagen at both mRNA and protein levels compared to norCM. In addition, hypoCM recruited more macrophages to the scald wound than norCM did. These findings suggest that hypoxia enhances the healing effect of pMSCs on scald wound partially through IGF-1.

Keywords: Placenta-derived mesenchymal stem cell, scald wound healing, insulin like growth factor-1, hypoxia, conditioned medium

Introduction

Despite the significant progress in burn treatment that has been made over the past decades, burn injury occurs with a high prevalence worldwide and still causes high morbidity and mortality even in the developed countries [1]. According to the World Health Organization (WHO), about 265,000 people die from burn injury every year. Hence, treatment of scald wound lays a heavy economic burden on the global health care system, yet effective therapies are very limited at present [2, 3].

Placenta-derived mesenchymal stem cells (pMSCs) are a new type of MSCs discovered in the recent years. pMSCs are pluripotent stem cells derived from the mesoderm during embryonic development. pMSCs possess similar biological features as bone marrow MSCs (BMSCs) [4-6], and also have their own unique advantages, such as rich sources, easy separation and expansion [7]. In addition, several studies have demonstrated that pMSCs would not form tumors *in vivo* [8]. Therefore, pMSCs are considered as a good source of stem cells with therapeutic potentials for many diseases [9-11].

So far, a number of studies have demonstrated a promising therapeutic effect of MSCs for the regeneration and repair of damaged tissues in many organs [12]. Like other cells utilized for cell-based therapy, MSCs may exert the therapeutic effect through interactions with the host cells in multiple ways. The mechanisms by which MSCs promote tissue repair have been debated for years. Currently, two leading theories explaining the therapeutic effects of MSCs are: 1) cell replacement theory: MSCs physically substitute for the lost cells in the damaged tissue; 2) paracrine theory: MSCs promote repair and regeneration of the damaged tissue by secreting a series of cytokines. Although a lot of evidences suggest that MSCs can migrate to the site of injury and differentiate into the type(s) of the damaged cells [13], only a small fraction of the MSCs eventually survived after engraftment [14, 15]. In fact, the small number of survived MSCs can hardly reconstitute the injured tissue or achieve functional recovery, and more studies suggest that MSCs participate in tissue repair through the paracrine network [16].

Scald is a common type of burn. Nowadays, the researches on MSC-based therapy for scald are mainly concentrated on BMSCs. Previous studies have shown that the hypoxic conditioned medium (hypoCM) of BMSCs can accelerate wound healing, which is largely related to the promotion effect of BMSC-derived hypoCM on the proliferation and migration of fibroblasts and vascular endothelial cells [17].

Our previous study demonstrated that hypoxia promoted secretion of insulin-like growth factor-1 (IGF-1) by pMSCs [18]. IGF-1 has been shown to promote proliferation and collagen synthesis in cardiac fibroblasts [19, 20], and it can also promote proliferation and migration of vascular endothelial cells [21]. It is known that both fibroblasts and vascular endothelial cells are important players in scald wound healing. Thus, whether pMSC-derived hypoCM has a therapeutic effect on scald wound healing is worth further study.

This study tested the hypothesis that the paracrine function of pMSCs is enhanced by hypoxia for promoting scald wound healing. pMSCs were cultured in normoxic (20% atmospheric O_2) or hypoxic (1% O_2) conditions, and the level of IGF-1 was measured. Conditioned medium of normoxia-treated and hypoxia-treated cells was collected, assessed for the effect on the proliferation and migration of fibroblasts, human umbilical vein endothelial cells (HUVECs) and macrophages *in vitro*, and applied to the scald wound on mice skin. In the meanwhile, anti-IGF-1 antibody (IGF-1Ab) was added in the conditioned medium to counteract the effect IGF-1 on scald wound healing. The results of this study demonstrate a significant impact of hypoxia on the paracrine activity of pMSCs in scald wound healing, and provide evidences for the potential application of pMSC-derived hypoCM as a cell-free therapy for burn injury.

Materials and methods

Ethics statement

This study was approved by the Ethics Committee of China Medical University (Liaoning, China). Human placentae were collected after delivery with the consent of the puerperae. The Balb/c mice used for scald wound healing experiment were 8-9 weeks old and weighed 20-25 g. All efforts were made to minimize animal suffering and the number of mice utilized.

Cell culture

Murine fibroblasts were isolated from the tails of neonatal Balb/c mice using a previously established method [22]. Murine macrophages were extracted from the peritoneal cavity fluids of Balb/c mice. The HUVEC cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and expanded by using a standard cell-culture protocol.

Isolation and passage of pMSCs

Placental tissues of cesarean full-term healthy newborns were obtained in sterile conditions. pMSCs were isolated as previously described [23], and cultured in high-glucose DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Bioind, Mazkeret Batya, Israel), 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA), 0.1 mM β-mercaptoethanol (Sigma, St. Louis, MO, USA) and 2 mM L-glutamine (Invitrogen). When reaching 90% confluence, the cells were harvested by trypsinization (Invitrogen) and re-plated in a new dish at a density of 5×10^5 per 10 cm dish. All experiments were performed with the third generation pMSCs.

Trypan blue staining

Cells were trypsinized and made into single cell suspension with PBS. The single cell suspension was mixed with trypan blue solution (2×) in a ratio of 1:1. The numbers of live and dead cells were counted under an optical microscope for three times. Dead cells were stained blue, and live cells were unstained and transparent.

Cartilage cell differentiation assay and collagen II staining

The medium for cartilage cell differentiation was high-glucose (4.5 g/ml) DMEM supplemented with 10 M dexamethasone (Sigma), 10 ng/ml recombinant human transforming growth factor-β1 (R&D, Valais, Switzerland), 6.25 μg/ml insulin (Sigma), 6.25 μg/ml transferin (Sigma), 50 μg/ml ascorbic acid (Sigma), 100 ng/ml IGF-1 (Biovision, Milpitas, CA, USA) and 5% FBS. The differentiation medium was replaced every three days. Three weeks later, the induced cells were fixed with 4% formalin and probed for collagen II by immunocytochemistry with mouse anti-human collagen II antibody (1:100; Boster, Wuhan, China) according to the manufacturer's instructions.

Endothelial cell differentiation assay and von Willebrand factor (vWF) staining

The medium for endothelial cell differentiation contained 50 ng/ml recombinant human vascular endothelial growth factor (rHuVEGF) (Sigma), 10 ng/ml basic fibroblast growth factor (rHubFGF) (Sigma), 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, 0.1 mM nonessential amino acids (MEM), 1% penicillin/streptomycin and 5% FBS. The differentiation medium was replaced every three days. After 8 d induction, the cells were fixed with 4% formalin and detected for the expression of vWF by immunocytochemistry with mouse anti-human vWF antibody (1:100, Maxim, Fuzhou, China) according to the manufacturer's instructions.

Flow cytometry

pMSCs were trypsinized, washed twice with PBS and suspended to a final concentration of 5×10^5 cells/ml. The cells were incubated for 30 min at 4°C with one of the following antibodies: anti-CD29-FITC, anti-CD44-FITC, anti-CD31-FITC, anti-CD34-APC, anti-CD45-FITC, and anti-HLA-DR-FITC (all from BD, Franklin Lakes, NJ, USA). Then the cells were washed with PBS, and analyzed on a FACSCalibur flow cytometer (BD).

Hypoxic treatment

Thermo Fisher 311 anaerobic incubator (Thermo, Waltham, MA, USA) was used to control the hypoxic condition of 1% oxygen (1% O_2, 5% CO_2 and 94% N_2). The second generation pMSCs were seeded at 2000 cells/cm^2 and cultured for seven days under either hypoxia or normoxia. When the third generation pMSCs reached approximately 70%-80% confluence, the cells and the conditioned medium from normoxia-treated pMSCs (norCM) or from hypoxia-treated pMSCs (hypoCM) were harvested on ice.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to detect IGF-1 in the conditioned medium. Conditioned medium was collected at different time points during hypoxic or normoxic treatment, and the level of IGF-1 was determined with an IGF-1 ELISA kit (BD) according to the manufacturer's instructions. The concentration was calculated by the regression analysis of the standard curve.

The level of hypoxia-inducible factor-1 (HIF-1) in the cell nucleus was also detected by ELISA. The nuclear extracts were obtained immediately after harvest by using a nuclear extraction kit (Active Motif, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. The level of HIF-1 in the nuclear extract was measured with a commercial HIF-1 ELISA kit (Active Motif) following the manufacturer's protocol. The results were normalized to the cell number.

Endothelial cell tube formation assay

HUVECs (3×10^3) were seeded into each well of a 96-well plate pre-coated with 50 μL Matrigel (BD Biosciences, Bedford, MA, USA), and cultured in Vehicle medium, norCM, hypoCM, norCM+IGF-1 or hypoCM+IGF-1Ab (0.25 μg/ml, R&D systems, Minneapolis, MN, USA) [24] for 6 h at 37°C in a humidified atmosphere containing 5% CO_2. Images were captured under a DMI4000B microscope (Leica, Solmser Gewerbepark, Germany) at 100× magnification. The number of tubes in 4 randomly

selected fields was counted with the aid of the Olympus Cell R imaging software as previously described [25].

Scratch wound healing assay

For the scratch wound healing assay, cells (5×10^5 cells per well) were seeded into six-well plates and cultured at 37°C in an atmosphere of 5% CO_2. When the cells reached confluence, a scratch was made by scraping the cell monolayer with a 200 μl pipette tip. The cells were washed with PBS and cultured in the indicated conditioned medium. Cell migration was assessed by measuring the movement of the cells into the scraped area. Images of wound closure were captured at 0 h and 6 h using an inverted microscope under 100× magnification.

Immunohistochemistry (IHC)

Scald skin specimens were fixed in 10% formalin and dehydrated through a series of graded ethanol washes. Dehydrated specimens were embedded in paraffin, cut into 5 μm sections and mounted onto albumin-coated slides. The sections crossing through the wound center were selected in order to obtain maximal wound area for evaluation. After dewaxation and rehydration, the sections were subjected to IHC analysis. Briefly, the rehydrated sections were incubated in 3% hydrogen peroxide (H_2O_2) for 10 min to inhibit the activity of endogenous peroxidase. Subsequently, the sections were rinsed with deionized water and incubated in antigen retrieval solution (Target Retrieval Solution, Dako North America Inc., Carpentaria, CA, USA) in a water bath at 98°C to unmask antigens. After a further wash in Tris-buffered saline (TBS) (Biocare Medical, Concord, CA, USA), the sections were blocked with 10% goat serum (Vector Laboratories, Burlingame, CA, USA) for 1 h, and incubated overnight at 4°C with one of the following primary antibodies: rabbit anti-F4/80 polyclonal antibody (1:100 dilution, Proteintech, Chicago, IL, USA), rabbit anti-collagen type I polyclonal antibody (1:200 dilution, Proteintech) and rabbit anti-collagen type III polyclonal antibody (1:100 dilution, Proteintech). Thereafter, the sections were washed with TBS and incubated with goat anti-rabbit IgG antibody (1:200 dilution, Vector Laboratories). The reactions were further developed via an avidin-biotin complex reaction with the appropriate reagents (Vector Laboratories). Labeled cells were observed under a phase-contrast BX51WI microscope (Olympus, Japan). Positive cells were counted in 4 fields at 400× magnification as previously described [26].

Reverse transcription-PCR and quantitative real-time PCR

RNA extraction, reverse transcription-PCR and quantitative real-time PCR were performed as previously described [27]. The primers used for PCR amplification of collagen I were: sense 5'-TCCAAAGGAGAGAGCGGTAA-3' and antisense 5'-GACCAGGGAGACCAAACTCA-3'. The primers for collagen III were: sense 5'-TTATAAACCACCCTCTTCCT-3', and antisense 5'-TATTATAGCACCATTGAGAC-3'. All measurements were performed in three replicate.

Statistical analysis

All experiments were repeated for three times. Results are expressed as mean ± standard error of the mean (SEM). Differences between two groups were analyzed with Student's t test, and differences between multiple groups were compared using ANOVA and LSD *post hoc* test, with values of $P < 0.05$ considered statistically significant. All data were processed using SPSS.11 analysis software (SPSS Inc, Chicago, IL, USA).

Results

Identification of pMSCs

Human pMSCs were prepared from the culture of human placental tissues. After 10 days of culture, a moderate amount of cells appeared around the tissues. The cells were passaged and the hematopoietic cells were removed during passaging. The third generation pMSCs were fibroblast-like and spiral-shaped in morphology (**Figure 1A**). Most pMSCs were positive for CD73, CD90 and CD105, a group of markers for definition of multipotent MSCs as adopted by the International Society for Cell Therapy (**Figure 1B**). Majority of pMSCs were negative for CD34 (progenitor/endothelial marker), CD45 and HLA-DR (leukocyte markers) (**Figure 1B**). pMSCs were further confirmed by their abilities of differentiating into chondrocytes and endothelial cells (**Figure 1C**). Induced cartilage cells and endothelial cells were character-

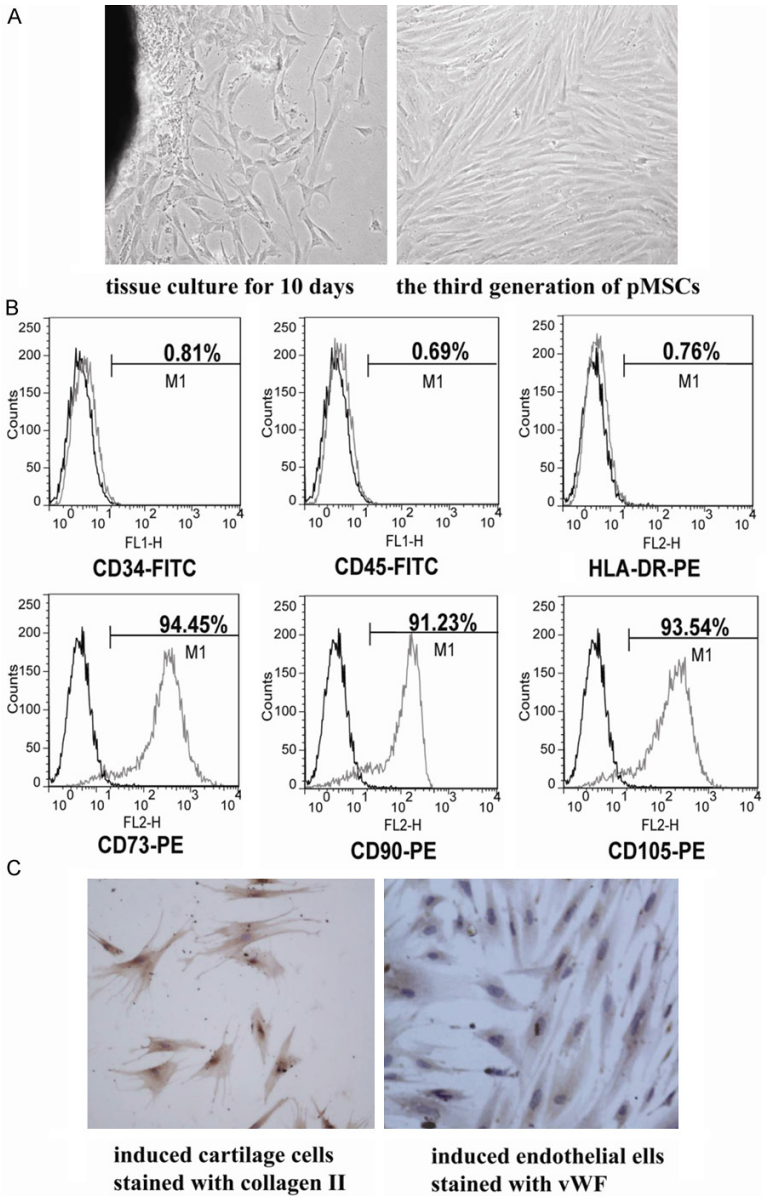

Figure 1. Isolation and identification of pMSCs. A: pMSCs was isolated from the placental tissue, and cultured *in vitro*. The third generation pMSCs were fibroblast-like in morphology, as visualized by phase-contrast microscopy (100× magnification). B: Flow cytometry analysis of MSC markers. Most pMSCs positively expressed CD73, CD90 and CD105, and negatively expressed CD34, CD45 and HLA-DR. C: Differentiation potential of pMSCs. pMSCs were induced to differentiate into cartilage cells and endothelial cells, which were identified by collagen II and vWF, respectively, via immunocytochemistry (400× magnification).

ized by collagen II and vWF, respectively. Only the cells that met the above criteria were used for the subsequent experiments.

Hypoxia upregulated HIF-1 and IGF-1 in pMSCs

pMSCs were cultured in hypoxia or normoxia for seven days, and the levels of activated HIF-1 and IGF-1 were detected by ELISA. As shown in **Figure 2A**, the level of activated HIF-1 in hypoxic pMSCs was profoundly higher than that in normoxic pMSCs after 7 d treatment ($P < 0.05$). Moreover, the content of secreted IGF-1 increased with time under both normoxia and hypoxia, and IGF-1 secretion was significantly enhanced in hypoxia as compared to normoxia from day 3 ($P < 0.05$; **Figure 2B**).

HypoCM of pMSCs promoted proliferation of fibroblasts and HUVECs via IGF-1

Trypan blue staining was used to detect viable cells. Fibroblasts or HUVECs were cultured in vehicle medium, norCM, hypoCM, hypoCM+IGF-1Ab or norCM+IGF-1, respectively, for five days. The numbers of fibroblasts (**Figure 3A**) and HUVECs (**Figure 3B**) in norCM, hypoCM, hypoCM+IGF-1Ab and norCM+IGF-1 were significantly greater than the respective controls cultured in the vehicle medium ($p < 0.05$). HypoCM and norCM+IGF-1 significantly promoted cell proliferation as compared with norCM ($P < 0.05$). In addition, compared with hypoCM, hypoCM+IGF-1Ab significantly decreased the number of viable cells ($P < 0.05$).

pMSC-derived hypoCM promoted migration of HUVECs and fibroblasts via IGF-1

Cell migration is required for optimal tissue repair. Here, the effect of IGF-1 on cell migration was first assessed by *in vitro* scratch wound healing assay. As shown in **Figure 4A**, HUVECs that were cultured in norCM, hypoCM, hypoCM+IGF-1Ab and norCM+IGF-1 migrated faster than those cultured in the vehicle medium ($P < 0.05$), and the rate of wound closure

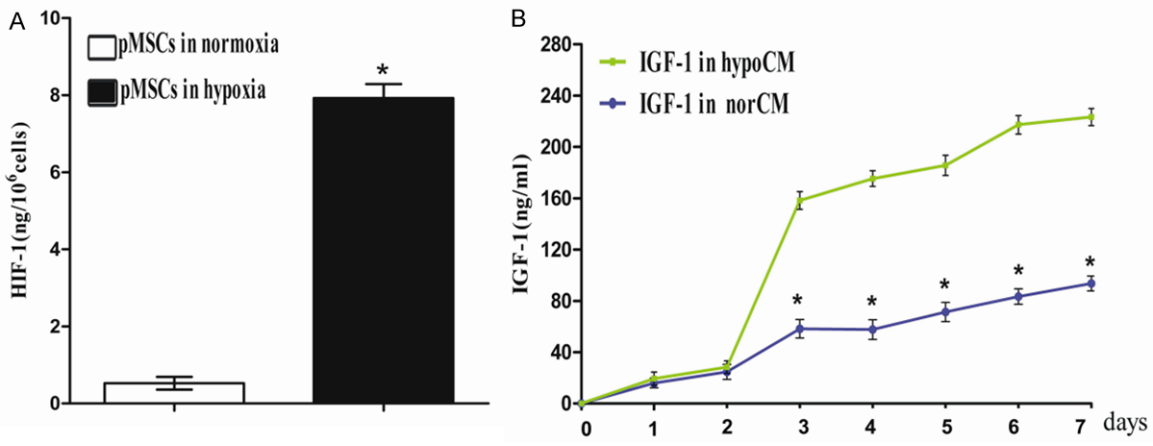

Figure 2. HIF-1 activation and IGF-1 secretion in pMSCs under normoxia and hypoxia. A: The level of activated HIF-1 in the nucleus of pMSCs in response to hypoxia or normoxia was determined by ELISA. *$P < 0.05$ versus pMSCs in normoxia. B: The level of secreted IGF-1 in the conditioned medium of pMSCs under hypoxia or normoxia. *$P < 0.05$ versus pMSCs in normoxia.

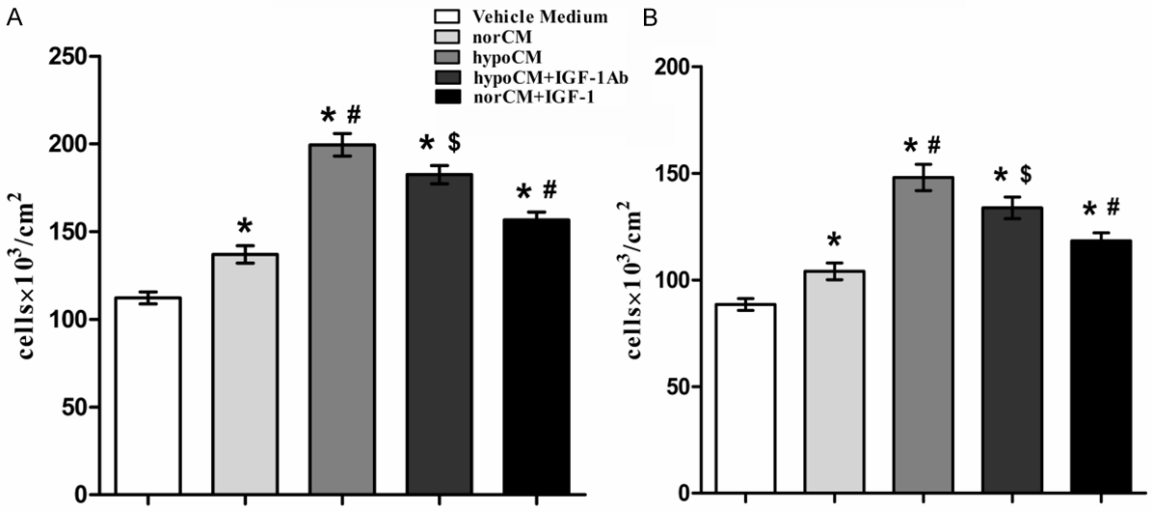

Figure 3. IGF-1 in hypoCM promotes proliferation of fibroblasts and HUVECs. Equal numbers of (A) fibroblasts or (B) HUVECs were cultured in vehicle medium, norCM, hypoCM, hypoCM+IGF-1Ab or norCM+IGF-1, respectively. Four days later, the number of living cells was determined by trypan blue staining. *$P < 0.05$ versus vehicle medium; #$P < 0.05$ versus norCM; $$P < 0.05$ versus hypoCM.

was higher in hypoCM- and norCM+IGF-1-treated HUVECs as compared with norCM-treated cells ($P < 0.05$). When IGF-1Ab was added into hypoCM, HUVECs migrated at a significantly lower rate ($P < 0.05$; **Figure 4A**). Likewise, norCM, hypoCM, hypoCM+IGF-1Ab and norCM+IGF-1 accelerated fibroblast migration compared with those in the vehicle medium ($P < 0.05$), and hypoCM displayed greater potency in promoting fibroblast migration than norCM ($P < 0.05$; **Figure 4B**). Moreover, migration of the fibroblasts was significantly suppressed in the presence of IGF-1Ab compared with the cells in hypoCM ($P < 0.05$; **Figure 4B**). In addition, migration of the macrophages were accelerated when the cells were cultured in norCM, hypoCM, hypoCM+IGF-1Ab and norCM+IGF-1, compared with those in the vehicle medium ($P < 0.05$; **Figure 4C**). Intriguingly, IGF-1Ab did not counteract hypoCM-enhanced migration of macrophages as it did in HUVECs and fibroblasts.

pMSCs-derived hypoCM enhanced neovascularization of HUVECs through IGF-1

Angiogenesis is an important physiological process during scald wound healing. HUVECs were

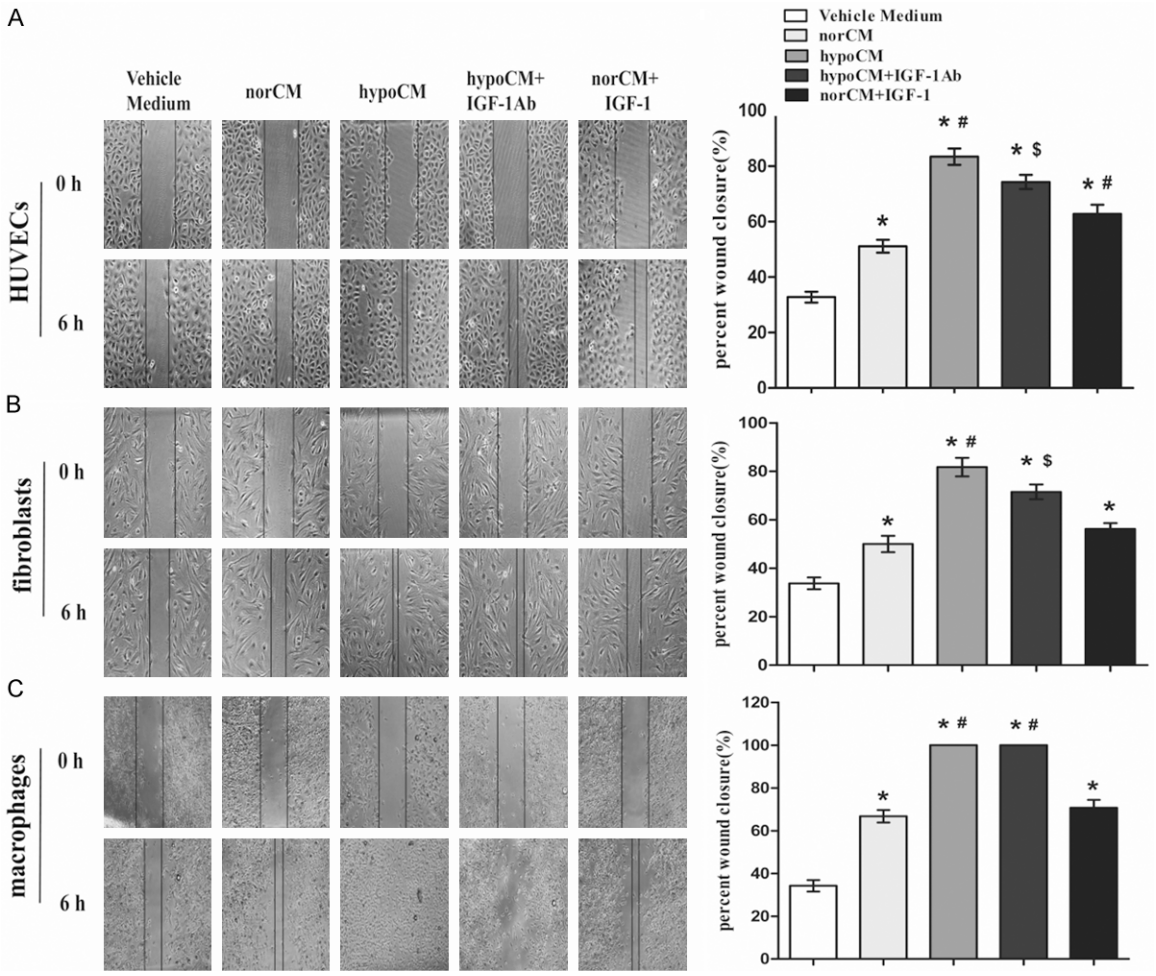

Figure 4. HypoCM promotes migration of HUVECs, fibroblasts and macrophages via IGF-1. The migration of (A) HUVECs, (B) fibroblasts and (C) macrophages was assessed by *in vitro* scratch wound healing assay. A scratch was made on the cell monolayer, and the cells were cultured in vehicle medium, norCM, hypoCM, hypoCM+IGF-1Ab and norCM+IGF-1, respectively. The images were captured immediately after scratching and 6 h later, and the percentage of wound closure was calculated accordingly. *$P < 0.05$ versus vehicle medium; #$P < 0.05$ versus norCM; $$P < 0.05$ versus hypoCM.

planted in matrigel matrix and cultured in the indicated conditioned medium. HUVECs formed significantly more tubes when cultured in norCM, hypoCM, hypoCM+IGF-1Ab and norCM+IGF-1 as compared with those cultured in the vehicle medium ($P < 0.05$; **Figure 5**). Compared with norCM, hypoCM and norCM+IGF-1 further promoted tube formation ($P < 0.05$). Moreover, addition of IGF-1Ab into hypoCM significantly decreased the number of tubes as compared to hypoCM ($P < 0.05$).

pMSCs-derived hypoCM accelerated scald wound healing in mice via IGF-1

To evaluate the effect of IGF-1 on scald wound healing *in vivo*, the indicated conditioned medium (100 μl) were topically applied to the scald wound on mouse skin. The wound size was measured on post-scald day 7. As shown in **Figure 6**, norCM, hypoCM, hypoCM+IGF-1Ab and norCM+IGF-1 significantly accelerated wound closure as compared with the vehicle medium ($P < 0.05$). The relative wound size was smaller after treatment with hypoCM or norCM+IGF-1 as compared with that treated with norCM ($P < 0.05$). By contrast, IGF-1Ab in hypoCM delayed wound healing as compared with hypoCM alone ($P < 0.05$).

pMSCs-derived hypoCM reduced collagen generation in mouse scald skin

To detect the effects of the conditioned media on collagen production during scald wound

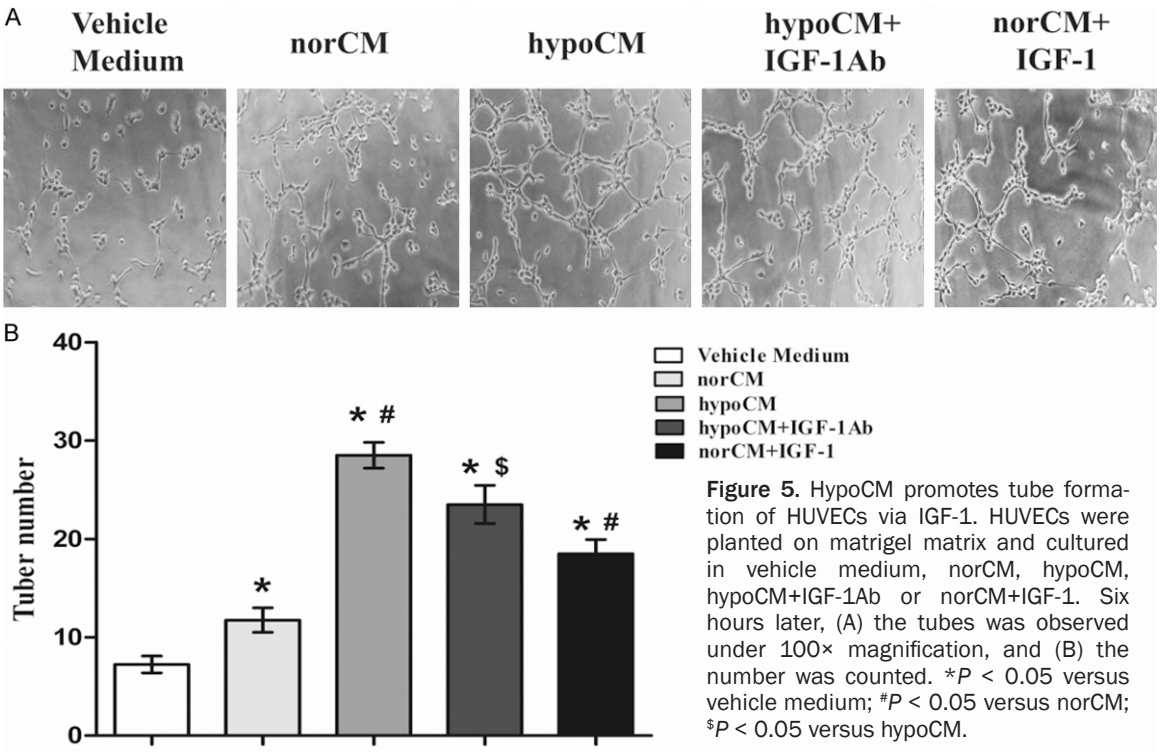

Figure 5. HypoCM promotes tube formation of HUVECs via IGF-1. HUVECs were planted on matrigel matrix and cultured in vehicle medium, norCM, hypoCM, hypoCM+IGF-1Ab or norCM+IGF-1. Six hours later, (A) the tubes was observed under 100× magnification, and (B) the number was counted. *P < 0.05 versus vehicle medium; #P < 0.05 versus norCM; $$P$ < 0.05 versus hypoCM.

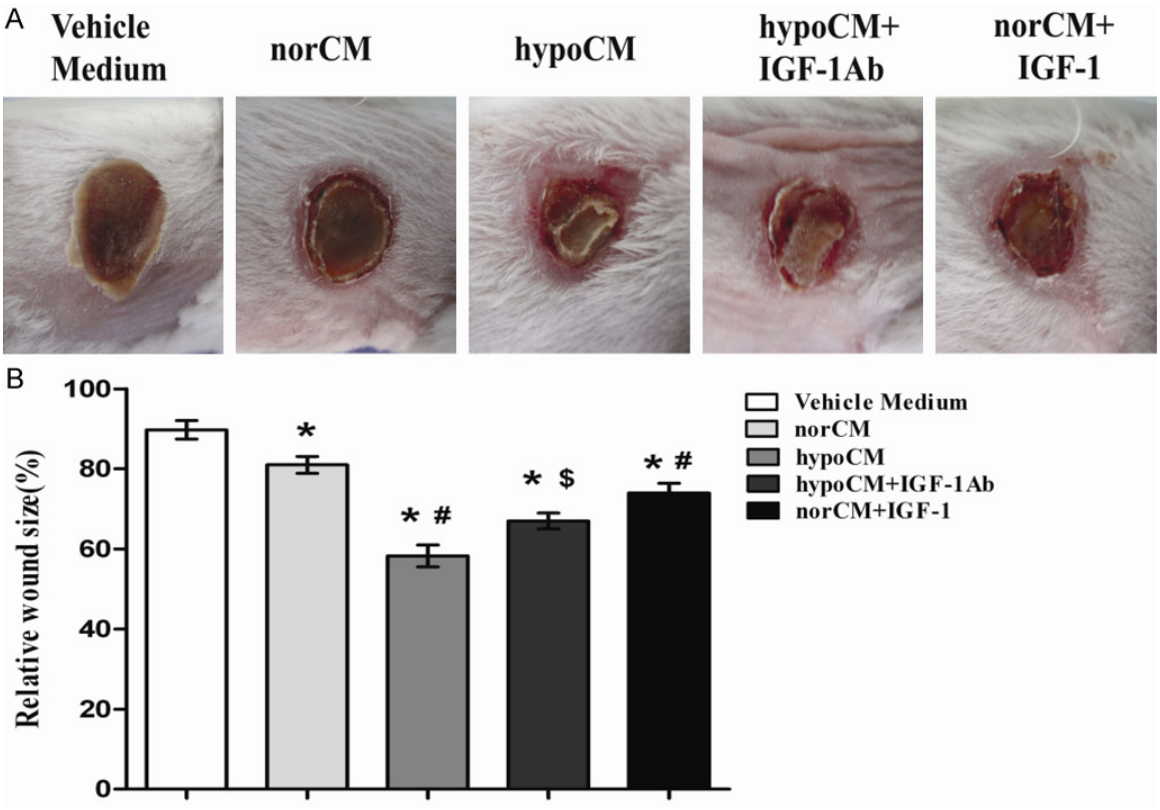

Figure 6. HypoCM and IGF-1 accelerates scald wound healing. The scald model was established on mouse skin with boiling water, and the wounds were treated with the indicated medium every day (n = 6 per group). Seven days later, dimension and thickness of the wounds were measured, and the relative wound size was calculated. *P < 0.05 versus vehicle medium; #P < 0.05 versus norCM; $$P$ < 0.05 versus hypoCM.

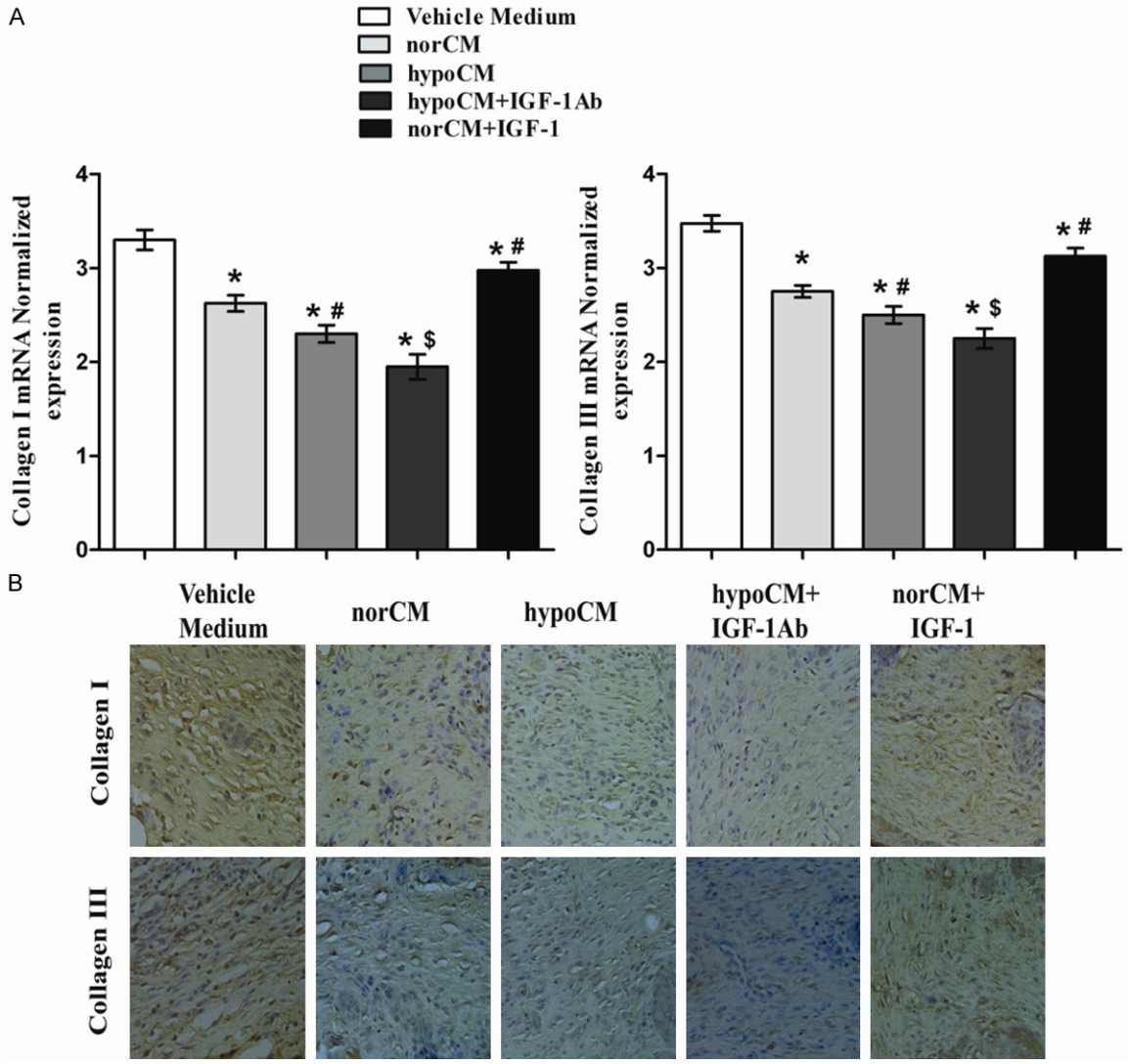

Figure 7. HypoCM reduces synthesis of type I and type III collagen in the scald wound. A: Relative mRNA expression of type I and type III collagen in the scald wound. B: IHC staining of type I and type III collagen in the wound (400× magnification). *$P < 0.05$ versus vehicle medium; #$P < 0.05$ versus norCM; $$P < 0.05$ versus hypoCM. (n = 6 per group).

healing, IHC staining and real-time PCR were performed to detect the expression of type I and type III collagen in the scald skin at protein and mRNA levels. As shown in **Figure 7A**, the mRNA level of type I and type III collagen in norCM-, hypoCM-, hypoCM+IGF-1Ab- and norCM+IGF-1-treated scald wound were lower than that in vehicle medium-treated wound ($P < 0.05$), and the mRNAs of type I and type III collagen in hypoCM-treated wound were significantly reduced compared with norCM-treated wound ($P < 0.05$). When IGF-1Ab was added into hypoCM, mRNA of type I and type III collagen was further decreased ($P < 0.05$). Moreover, norCM+IGF-1 increased the production of type I and type III collagen as compared to norCM ($P < 0.05$). These observations were further confirmed by the IHC results (**Figure 7B**).

pMSCs-derived hypoCM promoted macrophage migration to the scald wound in vivo

Macrophages are generally considered beneficial for scald skin healing. Here, we quantified the macrophages that were recruited to the scald wound after treatment by IHC staining for F4/80, a typical marker of mature macrophages. NorCM-, hypoCM-, hypoCM+IGF-1Ab-

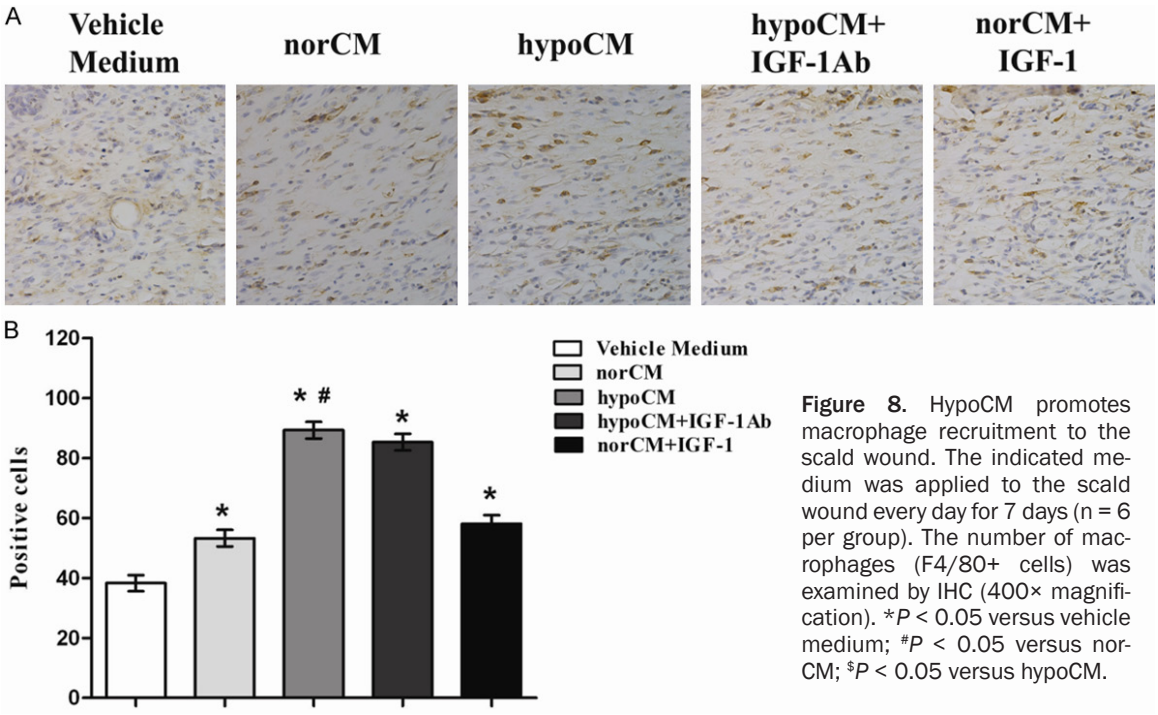

Figure 8. HypoCM promotes macrophage recruitment to the scald wound. The indicated medium was applied to the scald wound every day for 7 days (n = 6 per group). The number of macrophages (F4/80+ cells) was examined by IHC (400× magnification). $^{*}P < 0.05$ versus vehicle medium; $^{\#}P < 0.05$ versus norCM; $^{\$}P < 0.05$ versus hypoCM.

and norCM+IGF-1-treated wounds recruited an increased number of F4/80+ cells as compared with vehicle medium-treated wounds ($P < 0.05$, **Figure 8**). HypoCM further promoted microphage migration to the wound site compared with norCM ($P < 0.05$). There was no significant difference in the number of macrophages between hypoCM-treated wound and hypoCM+IGF-1Ab-treated wound, and it was the same between norCM and norCM+IGF-1 treatment, suggesting that IGF-1 may not play a role in macrophage recruitment during scald wound healing.

Discussion

Scald is a kind of tissue damage with localized necrosis. Scald wound healing involves a series of physiological processes of regeneration and reconstruction, and it is generally divided into three stages: inflammatory stage, proliferative stage and remodeling stage. Scald wound healing is executed by several types of cells including fibroblasts, vascular endothelial cells and macrophages.

The primary event in the inflammatory stage is the infiltration of neutrophils and macrophages to the wound site. The main features of the proliferation stage include migration, proliferation and differentiation of fibroblasts, as well as synthesis, secretion and deposition of extracellular matrix. The remodeling stage mainly involves maturation of the granulation tissue and its subsequent transformation into a scar. The process of scald wound healing depends on the cooperation of various cells, and is also influenced by direct or indirect actions of multiple cytokines. Hence, scald wound healing is a dynamic process of complex interactions between cells, cytokines and extracellular matrix.

pMSCs are a new type of MSCs discovered in the recent years. Because of their superior features like rich sources, easy separation and amplification *in vitro*, pMSCs are considered to be an attractive source of MSCs. Our previous study demonstrated that IGF-1 section in pMSCs was upregulated in hypoxia [18]. IGF-1 has been shown to promote proliferation and migration of fibroblasts and vascular endothelial cells [19-21], which are key players in scald wound healing. Thus, we speculated that pMSC-derived hypoCM may promote scald wound healing via a high level of secreted IGF-1.

At present, BMSC is the most common model for studying the therapeutic effect of MSCs on

scald skin. A recent study by Chen *et al.* demonstrated that BMSC-derived hypoCM could promote wound healing in mice [17], yet there are limited literature about the effect of pMSCs on scald wound healing.

In the present study, we successfully isolated pMSCs from human placenta and identified MSC surface markers on the isolated cells. Hypoxic treatment enhanced activation of HIF-1 and secretion of IGF-1 in pMSCs. It is known that scald skin healing is a process during which the necrotic tissue is replaced by the granulation tissue comprising of new capillaries, fibroblasts and inflammatory cells. Our results showed that pMSC-derived norCM and hypoCM promoted proliferation of fibroblasts and HUVECs, and hypoCM further augmented cell proliferation. When IGF-1Ab was added into hypoCM, hypoCM-stimulated cell proliferation was slowed down. By contrast, when IGF-1 was added into norCM, cell proliferation was accelerated. These results suggest that hypoCM has a strong potency in promoting the proliferation of fibroblasts and HUVECs at least partially attributed to IGF-1.

The *in vitro* scratch wound healing assay showed that both norCM and hypoCM enhanced migration of fibroblasts or HUVECs compared with the vehicle medium, and hypoCM displayed a stronger potency than norCM. In the presence of IGF-1Ab, hypoCM-enhanced migration of fibroblasts and HUVECs was attenuated, whereas external IGF-1 increased the mobility of HUVECs. Therefore, it can be concluded that IGF-1 in hypoCM contributed to the enhanced migration of fibroblasts and HUVECs.

It is known that macrophages participate throughout the whole process of scald wound healing by phagocytosing the necrotic cells and pathogenic microorganisms and secreting a variety of cytokines that stimulate cell proliferation, promote angiogenesis and accelerate collagen deposition. The scratch wound assay showed that pMSC-derived norCM and hypoCM promoted the migration of macrophages, and macrophages migrated faster in hypoCM than in norCM. However, there was no significant difference in the migration rate between hypoCM+IGF-1Ab- and hypoCM-treated macrophages, suggesting that it was not IGF-1, but other cytokines secreted by pMSCs under hypoxia, that was responsible for the hypoCM-enhanced macrophage migration.

During the process of scald wound healing, local vascular endothelial cells form new blood vessels through proliferation and migration. Angiogenesis is the basis for tissue repair because the new blood vessels supply oxygen, nutrients and bioactive substances for wound healing. Previous studies have shown that IGF-1 can promote angiogenesis by stimulating vascular endothelial cells to secrete VEGF which in turn acts on the vascular endothelial cells [28]. In this study, hypoCM significantly enhanced tube formation of HUVECs *in vitro*, whereas the number of newly formed tubes was significantly decreased in the presence of IGF-1Ab. By contrast, tube formation was markedly enhanced when IGF-1 was added in norCM. These results suggest that hypoCM promoted neovascularization partly because of the increased IGF-1 in hypoCM.

Abnormal tissue repair may occur as a result of persistent repair activities in severe scald. Excessive collagen deposition in the dermis of the scald skin can replace the normal tissue and eventually form a scar. Despite many methods for inhibiting scar formation at present, there is still a debate on promoting or inhibiting scar formation for scald wound healing. Excessive inhibition of scar formation may affect scald wound healing, but utilization of cytokines to promote wound healing can increase the risk of scar formation. Our *in vivo* assays showed that the scald wound treated with hypoCM healed faster than that treated with norCM. When IGF-1Ab was added into hypoCM, the healing effect of hypoCM was attenuated, whereas additional IGF-1 enhanced the healing effect of norCM. The results suggest that hypoCM promotes scald wound healing via IGF-1.

The study by Tokudome *et al.* showed that IGF-1 promoted pulmonary fibrosis by increasing collagen synthesis in fibroblasts [29]. In our study, the contents of both type I and type III collagen were decreased when IGF-1Ab was added into hypoCM, whereas the levels of both types of collagen were increased when IGF-1 was added, implying that IGF-1 promoted collagen synthesis during scald wound healing. Considering our previous data that hypoCM enhanced IGF-1 secretion in pMSCs, we speculated that hypoCM-treated scald skin would produce more collagen than norCM-treated scald skin. Surprisingly, we got the opposite results. Collagen content was lower in hypoCM-

treated scald skin than that in norCM-treated scald skin at both mRNA and protein levels. HypoCM inhibited, instead of promoting, collagen synthesis in scald skin. Previous studies have shown that hypoxia stimulated pMSCs to secrete many types of cytokines, including pro-inflammatory cytokines, anti-inflammatory cytokines and growth factors. Pro-inflammatory cytokines and growth factors promote wound healing by enhancing collagen synthesis, whereas anti-inflammatory cytokines such as IL-10 can inhibit inflammation and reduce collagen production. Hence, our results showed that hypoCM inhibited type I and type III collagen synthesis in the sacld skin, which was the final outcome of combined actions of multiple cytokines secreted by pMSCs in response to hypoxia.

In summary, hypoxia stimulated IGF-1 secretion in pMSCs, and IGF-1 contributed to hypoCM-accelerated scald would healing by promoting cell proliferation (fibroblasts and HUVECs), cell migration (fibroblasts and HUVECs), and angiogenesis (HUVECs). Moreover, hypoCM could also inhibit excessive collagen synthesis and recruit macrophages in the scald skin. The healing effect of hypoCM is attributed to combined actions of multiple cytokines in hypoCM, and our study only illustrates one of them. The roles of other cytokines in hypoCM and their interaction during scald wound healing need to be explored in future studies. Since the application of pMSC-derived conditioned medium does not involve the problem of immune rejection, and the production, storage, packaging and transportation of conditioned medium are relatively easy, pMSC-derived conditioned medium has a promising prospect in clinical application as a regenerative medicine.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yanqiu Yu, Department of Pathophysiology, College of Basic Medical Science, China Medical University, 77 Puhe Road, Shenyang 110122, People's Republic of China. Tel: +8602422816021; E-mail: yanqiu_yqy@sina.com

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