Int J Clin Exp Pathol 2016;9(1):10-21
www.ijcep.com /ISSN:1936-2625/IJCEP0017502

Original Article

Hypoxic conditioned medium of placenta-derived mesenchymal stem cells inhibits TGF-β1-induced up-regulation of collagens and differentiation of skin fibroblasts to myofibroblast through IL-10

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Received October 8, 2015; Accepted November 20, 2015; Epub January 1, 2016; Published January 15, 2016

Abstract: Scar formation affects the quality of people's daily lives, and in this study we explored whether hypoxic conditioned medium of placenta-derived mesenchymal stem cells (PMSCs) has a protective effect on scar formation. Our study showed that hypoxic conditioned medium of PMSCs inhibited the transforming growth factor-β1 (TGF-β1)-induced up-regulation of collagens and differentiation of skin fibroblasts to myofibroblast. Mechanism study showed that the level of interleukin-10 (IL-10) was influenced by hypoxic culture condition of PMSCs and further study showed that IL-10 simulated the effects of hypoxic conditioned medium of PMSCs to inhibit TGF-β1-induced up-regulation of collagens and α-smooth muscle actin (α-SMA), whereas, the inhibition effects of hypoxic conditioned medium of PMSCs on TGF-β1-induced up-regulation of collagens and α-SMA was reversed by IL-10 antibody. Results of this study suggested that hypoxic conditioned medium of PMSCs performed a protective effect against scar formation through the regulation of IL-10. This study provides further evidence for the possible use of PMSCs as a promising method to treat wounds.

Keywords: Hypoxic conditioned medium, placenta-derived mesenchymal stem cells, transforming growth factor-β1, interleukin-10, scar formation, skin fibroblasts

Introduction

Wound repair is a complex process. In this process, excessive repair results in scar formation. Scars will cause problems in tissue growth and functions, and affect the quality of people's daily lives seriously. In the process of wound repair, skin fibroblasts play important roles. Skin fibroblasts produce large amounts of extracellular matrixes, adhesion molecules and growth factors and contribute to a variety of cell complexes, thus promoting wound repair and maintaining the integrity of skin [1]. Skin fibroblasts also differentiate to contractile myofibroblasts which accelerate wound closure.

Mesenchymal stem cells (MSCs) are similar to stem cells which have the capability to differentiate to multiple types of cells. In the process of wound healing, MSCs play important roles. MSCs can accelerate wound closure and re-epithelialization and reduce scar tissues [2]. MSCs derived from bone marrow and adipose showed beneficial effects on wound healing [3, 4]. Placenta-derived mesenchymal stem cells (PMSCs) have similar characteristics but more easy to be obtained and without invasive operations. PMSCs also showed a beneficial effect on wound repair [5]. Transplanted PMSCs localized in the wounded areas, differentiated to various types of skin cells, and also secreted large amounts of growth factors promoting angiogenesis [5].

Transforming growth factor-β1 (TGF-β1) is a multiple-functional cytokine. TGF-β1 has effects

on the proliferation, differentiation of cells, and regulates tissue remodeling and wound repair. TGF-β1 promotes synthesis of extracellular matrix (ECM) and inhibits the activities of matrix metallopeptidases (MMPs) [6, 7]. TGF-β1 also promotes the differentiation of fibroblast to myofibroblast, which plays an important role in scar formation [7].

Our previous study showed that hypoxia enhanced the protective effects of PMSCs on wound repair [8]. However, whether hypoxic PMSCs have beneficial effects on scar formation was still unknown. In the present study, we explored the effects of hypoxic conditioned medium of PMSCs on scar formation as well as the underlying mechanism. This study provides further evidence for the use of hypoxic PMSCs in the treatment of wounds.

Materials and methods

Isolation, culture, and identification of PMSCs

The placentas were cut into small pieces in sterile conditions, attached to the surface of dishes pro-coated with fetal bovine serum (FBS, Hyclone, Logan, UT, USA), and then incubated at 37°C for 12 h. Then Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) with 10% FBS was added to cells. PMSCs were harvested after cells migrated out of tissues and seeded in new dishes. The isolated PMSCs were cultured in DMEM with 10% FBS and maintained in a humid atmosphere at 37°C with 5% CO_2. Cells at passage 3 were used for the identification of PMSCs. For the identification of the isolated PMSCs, the isolated cells were harvested and incubated with fluorescein isothiocyanate (FITC)-conjugated antibodies against CD29, CD31, CD34, CD44, CD45 and HLA-DR (eBioscience, San Diego, CA, USA) in the dark at room temperature for 30 min. Then the stained cells were analyzed using flow cytometry.

Cell culture

Human skin fibroblast HFF-1 was obtained from Type Culture Collection Center of Chinese Academy of Science (Shanghai, China). HFF-1 cells were cultured in DMEM with 10% FBS and maintained in a 37°C incubator with a humid atmosphere and 5% CO_2.

Hypoxia treatment

The isolated PMSCs were cultured in DMEM with 10% FBS and maintained in a normoxic condition (20% O_2) or hypoxic condition (5% O_2 or 1% O_2) respectively for 72 h. Then cells were collected for the detection of interleukin-10 (IL-10) using quantitative real-time polymerase chain reaction (qRT-PCR), western blot, and enzyme-linked immunosorbent assay (ELISA). The normoxic and hypoxic conditioned medium of PMSCs was also collected for subsequent experiments.

TGF-β1 treatment

HFF-1 cells were cultured in normal medium, normoxic conditioned medium, or hypoxic conditioned medium and treated with 5 ng/ml TGF-β1 for 48 h, then the expression level of collagen I and collagen III were detected by western blot, and the level of α-smooth muscle actin (α-SMA) was detected by qRT-PCR, western blot and Immunofluorescence.

IL-10 and IL-10 antibody treatment

HFF-1 cells were cultured in i) normal medium, ii) normal medium plus 5 ng/ml TGF-β1, iii) normoxic conditioned medium plus 5 ng/ml TGF-β1, iv) normoxic conditioned medium plus 5 ng/ml TGF-β1 and 10 ng/ml IL-10, v) hypoxic conditioned medium plus 5 ng/ml TGF-β1, vi) hypoxic conditioned medium plus 5 ng/ml TGF-β1 and IL-10 antibody, for 48 h. Then the expression level of collagen I, collagen III, and α-SMA was detected by western blot.

qRT-PCR

Total RNA was extracted using a High-purity Total RNA Extraction Kit (BioTeke, Beijing, China) according to the protocol and reverse-transcribed to cDNA using M-MLV reverse-transcriptase (BioTeke) and Oligo$(dT)_{15}$. Then the mRNA levels of IL-10 and α-SMA was measured by qRT-PCR using cDNA as templates and primers as following: forward primer for IL-10, CGATTTAGAAAGAAGCCCAA; reverse primer for IL-10, TCAACAGCTAGAAAGCGTGGT; forward primer for α-SMA, TCCCTTGAGAAGAGTTACGAGTT; reverse primer for α-SMA, ATGATGCTGTTGTAGGTGGTT; forward primer for β-actin, CTTAGTTGCGTTACACCCTTTCTTG; reverse primer for β-actin, CTGTCACCTTCACCGTTCCAGTTT.

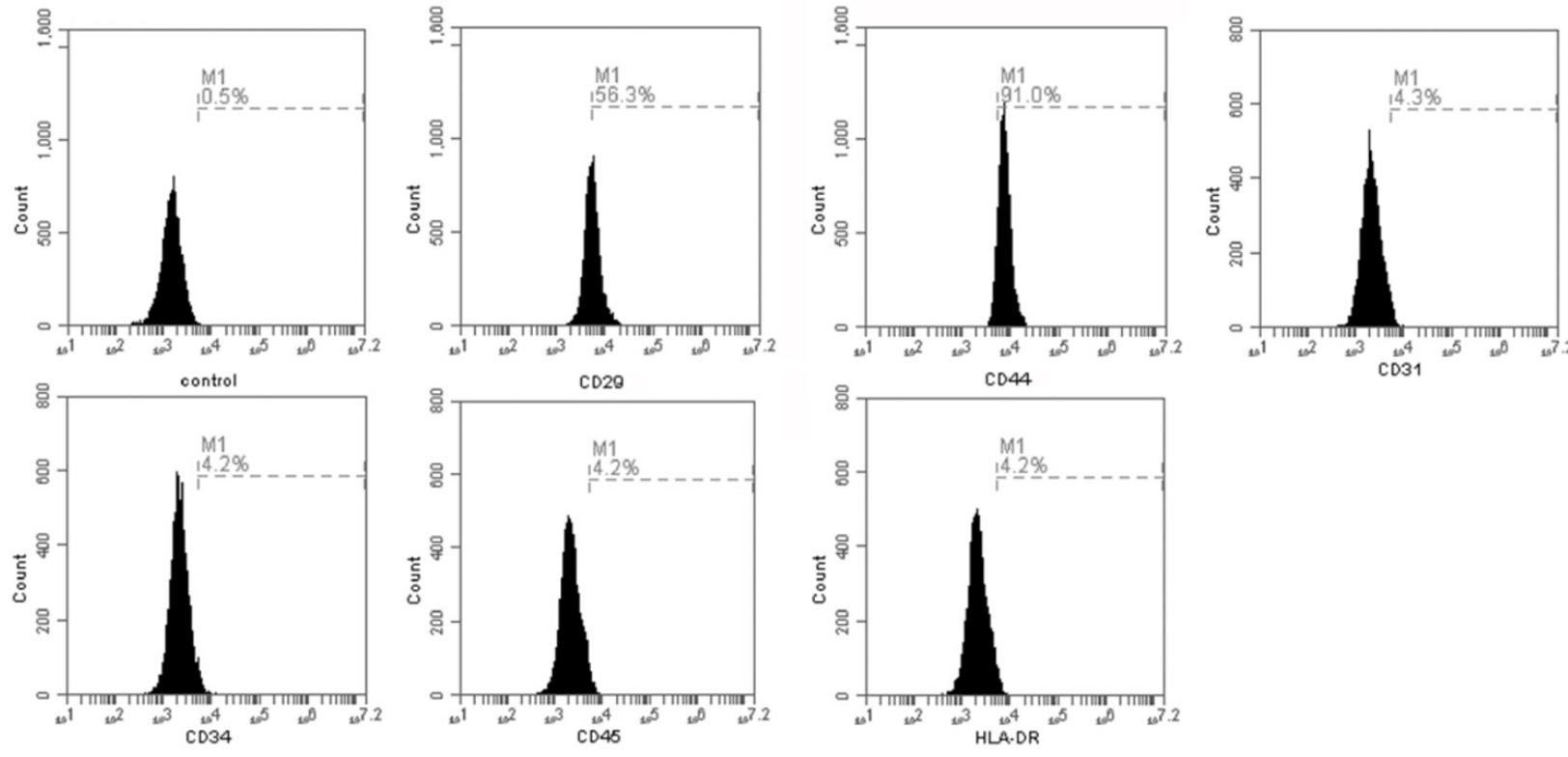


Figure 1. Identification of isolated PMSCs. The isolated cells were stained with CD29, CD31, CD34, CD44, CD45 and HLA-DR antibody and analyzed by flow cytometry.

The cycling parameters were 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s and 72°C for 30 s and then 4°C for 5 min. The relative mRNA levels of IL-10 and α-SMA were normalized to β-actin and calculated using $2^{-\Delta\Delta Ct}$ method [9].

Western blot

Total protein was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Haimen, China). The protein concentration was measured with an Enhanced BCA Protein Assay Kit (Beyotime). Thereafter, equal amount of protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then the separated protein was transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blockade with 5% skim milk, the PVDF membranes were incubated with primary antibodies against IL-10 (1:500, Bioss, Beijing, China), collagen I, collagen III, and α-SMA (1:400, Boster, Wuhan, China), and β-actin (1:1000, Santa Cruz, Dallas, TX, USA) at 4°C overnight. After washing with Tris-buffered saline with Tween (TBST), the PVDF membranes were incubated with corresponding secondary antibodies (1:5000, Beyotime) at 37°C for 45 min. The objective protein was visualized using ECL Detection System (7sea biotech, Shanghai, China) and the gray level was analyzed using Gel-Pro-Analyzer software.

ELISA

The levels of IL-10 in the cell medium of PMSCs cultured in normoxic or hypoxic condition were detected using an IL-10 ELISA Kit (WHB, Shanghai, China) according to the manufacturer's instruction.

Immunofluorescence

After treatment with normoxic or hypoxic conditioned medium and TGF-β1, skin fibroblasts were made into cell climbing. After fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100, the cell climbing were blocked with normal goat serum and incubated with primary antibody against α-SMA (1:200, Boster) at 4°C overnight. After washing with PBS, cell climbing was incubated with Cy3-labeled secondary antibody (1:200, Beyotime) at room temperature for 60 min in the dark. The cell nucleuses were counterstained with DAPI. Images of cells were captured under a fluorescence microscope with 400× magnification.

Statistical analysis

All experiments were performed three times and the results are presented as mean ± standard deviation (SD). Differences between each group were analyzed by one-way ANOVA and Bonferroni's Multiple Comparison. $P < 0.05$ was considered to be significant.

Results

Identification of PMSCs

The isolated PMSCs were first identified using flow cytometry. As shown in **Figure 1**, the isolated cells were CD29+, CD44+, CD31-, CD34-, CD45-, and HLA-DR-. These results demonstrated that the isolated cells were PMSCs.

Hypoxia enhanced the expression and secretion of IL-10

PMSCs were cultured in normoxic condition or hypoxic condition, and then the level of IL-10 was detected by qRT-PCR and western blot. Results of qRT-PCR showed that the mRNA level of IL-10 was increased significantly after culture in hypoxic condition, compared with that of cells cultured in normoxic condition (**Figure 2A**). Results of western blot also showed that, after culture in hypoxic condition, the protein level of IL-10 was elevated (**Figure 2B** and **2C**), which was consistent with the results of qRT-PCR. The level of IL-10 in the cell medium of PMSCs was also detected by ELISA. Results showed that, after culture in hypoxic condition, the level of IL-10 in cell medium was increased (**Figure 2D**), which indicated that the secretion of IL-10 was enhanced. These results demonstrated that hypoxia enhanced the expression and secretion of IL-10.

Hypoxic conditioned medium of PMSCs decreased the collagen level induced by TGF-β1

The levels of collagens have a close relationship with the formation of scars, and the effects of hypoxic conditioned medium of PMSCs on collagen levels induced by TGF-β1 was explored. Results of western blot showed that the levels of collagen I and collagen III were up-regulated

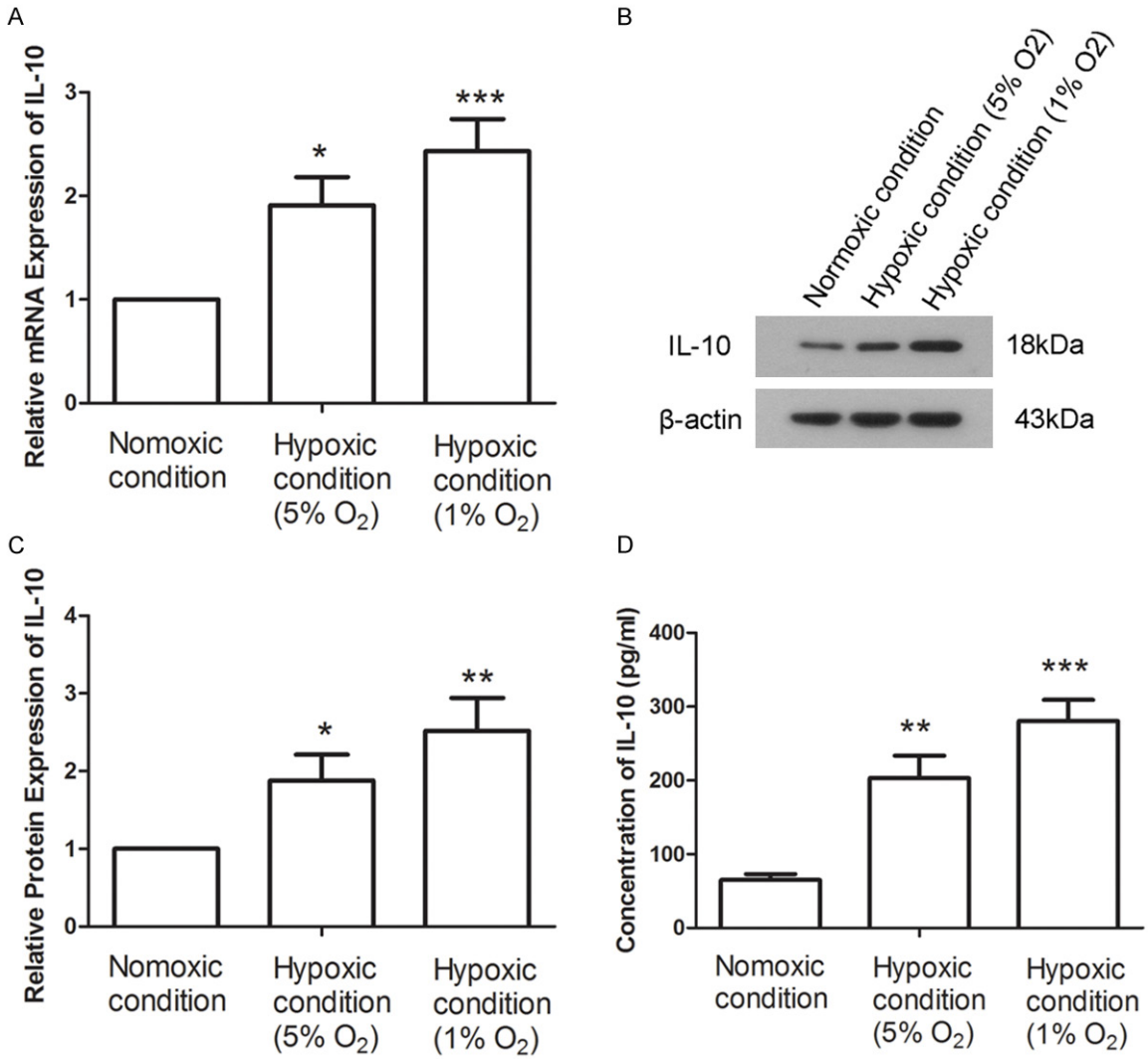


Figure 2. Hypoxia enhances the expression and secretion of IL-10. A. qRT-PCR was used to measure the mRNA level of IL-10 in PMSCs cultured in hypoxic condition. B, C. The protein level of IL-10 in PMSCs was detected by western blot after culture in hypoxic condition. β-actin was used as an internal reference. D. Concentration of IL-10 in the cell medium was detected after treatment with hypoxia. All experiments were repeated three times and the results are presented as mean ± SD. $P < 0.05$ was considered to be significant.

by TGF-β1. However, hypoxic conditioned medium reversed the up-regulation of collagen I and collagen III levels induced by TGF-β1 (**Figure 3**). These results suggested that hypoxic conditioned medium of PMSCs reduced collagen level induced by TGF-β1.

Hypoxic conditioned medium of PMSCs inhibited the differentiation of skin fibroblasts to myofibroblasts induced by TGF-β1

The differentiation of fibroblasts to myofibroblasts plays important roles in the formation of scars, and TGF-β1 has a close relationship with this differentiation process. α-SMA is a marker for differentiation of fibroblasts to myofibroblasts and the level of α-SMA was detected by qRT-PCR, western blot and immunofluorescence. Results of qRT-PCR showed that, after treatment with TGF-β1, the mRNA level of α-SMA was increased to 3.87±0.48-fold. However, after treatment with hypoxic conditioned medium of PMSCs, the mRNA level of α-SMA was decreased to 1.89±0.26-fold (**Figure 4A**). Similar results were also found in western blot, the protein level of α-SMA was increased after treatment with TGF-β1, but was reversed by hypoxic conditioned medium of PMSCs (**Figure**

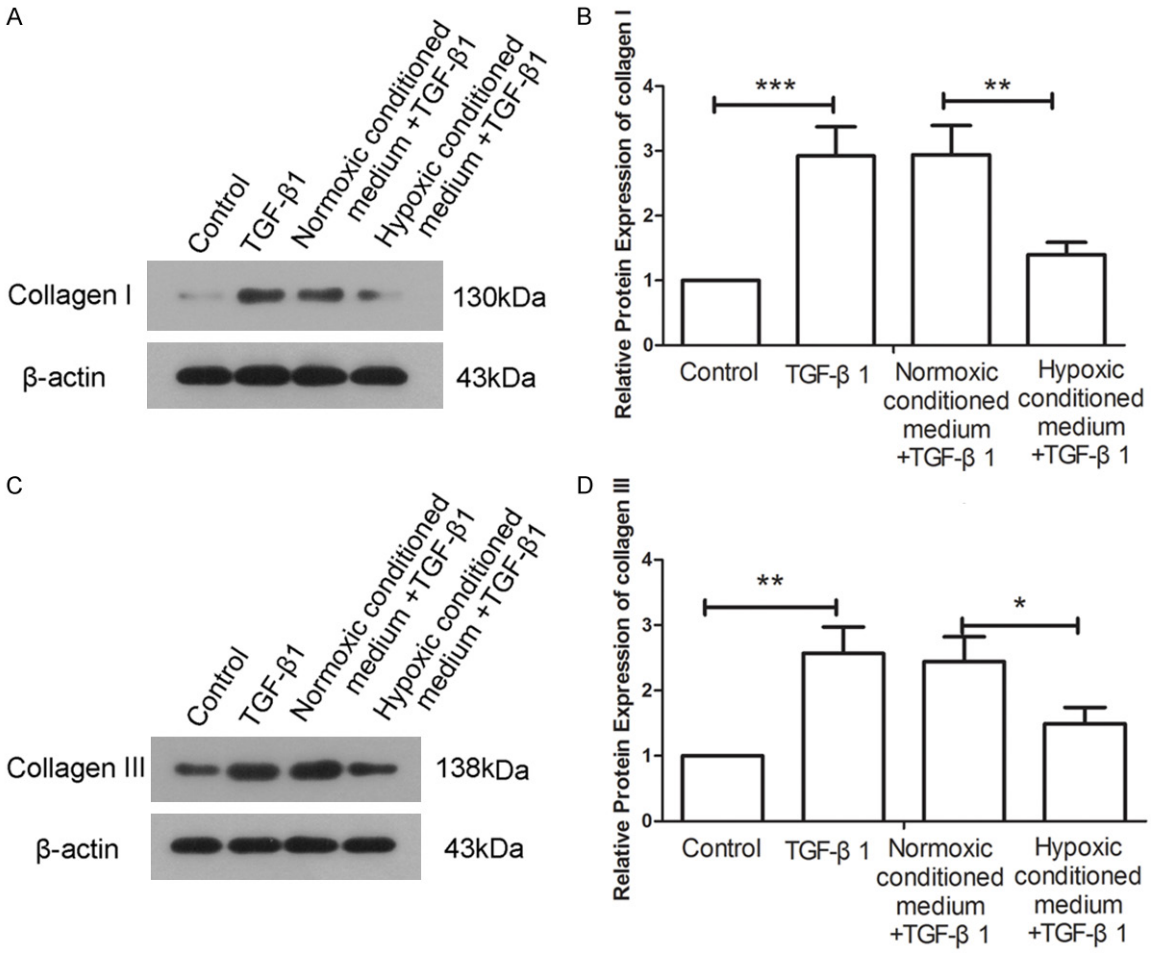


Figure 3. Hypoxic conditioned medium of PMSCs inhibits TGF-β1-induced up-regulation of collagen I and collagen III. A, B. Protein level of collagen I was detected by western blot after treatment with TGF-β1 plus normoxic or hypoxic conditioned medium of PMSCs. The relative protein level of collagen I was calculated using β-actin as an internal reference. C, D. The protein level of collagen III was measured using western blot and relative collagen III level was calculated. All experiments were repeated three times and typical results are presented. The results are shown as mean ± SD. $P < 0.05$ was considered to be significant.

4B and **4C**). Results of immunofluorescence showed that the expression and distribution of α-SMA was regulated by TGF-β1, but reversed by hypoxic conditioned medium of PMSCs (**Figure 4D**). These results demonstrated that hypoxic conditioned medium inhibited the differentiation of fibroblasts to myofibroblasts induced by TGF-β1.

IL-10 was involved in the protective role of hypoxic conditioned medium of PMSCs

To further explore whether IL-10 was involved in the function of hypoxic PMSCs, IL-10 and IL-10 antibody was introduced into our study. As shown in **Figure 5**, after treatment with TGF-β1, the protein levels of collagen I, collagen III and α-SMA were increased. However, when IL-10 was introduced into our study, the protein levels of collagen I, collagen III and α-SMA were decreased. This indicated that treatment with IL-10 can simulate the effects of hypoxic conditioned medium of PMSCs. IL-10 antibody was also introduced into our study. Results showed that, after treatment with IL-10 antibody, levels of collagen I, collagen III and α-SMA was increased compared with those of cells treated with hypoxic conditioned medium of PMSCs plus TGF-β1. These results indicated that the inhibition effect of hypoxic conditioned medium of PMSCs on TGF-β1-induced up-regulation of collagen I, collagen III and α-SMA was reversed by IL-10 antibody. Results of immunofluores-

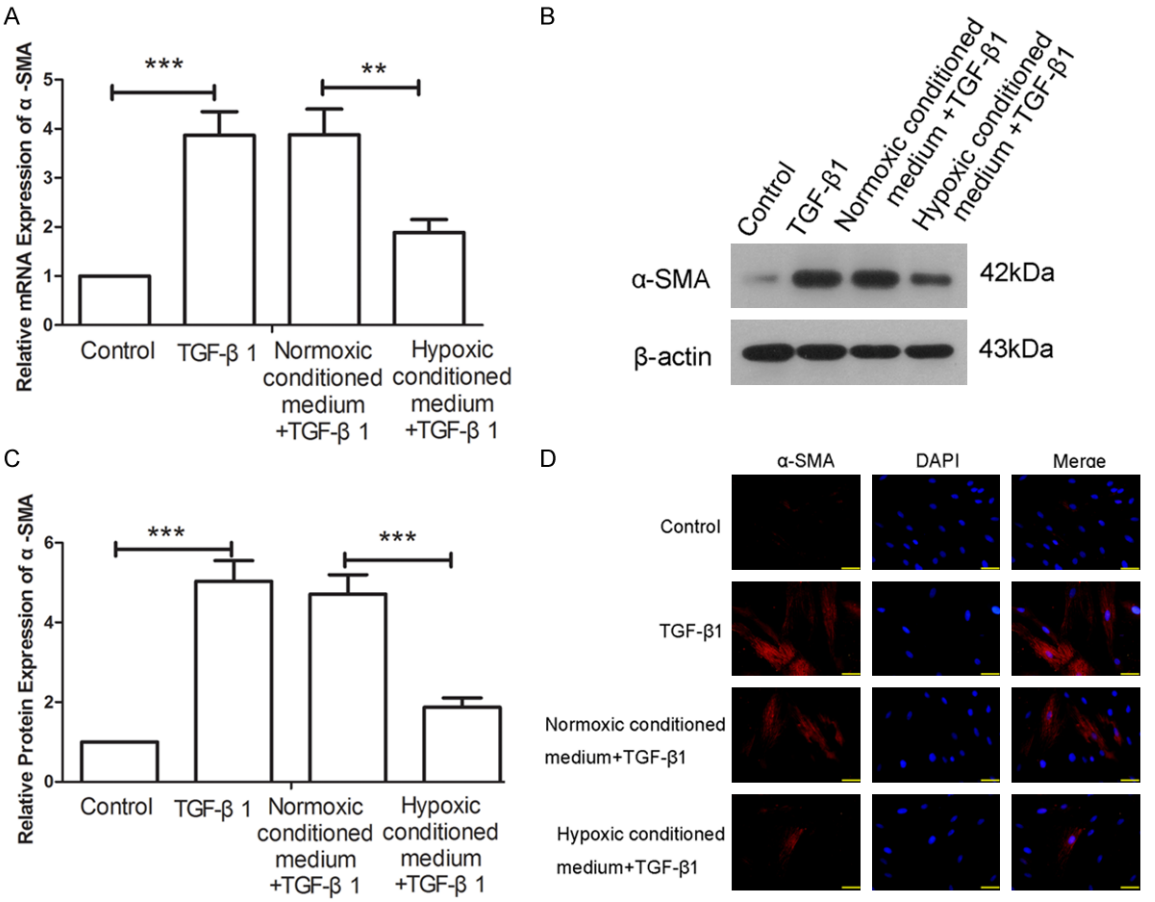


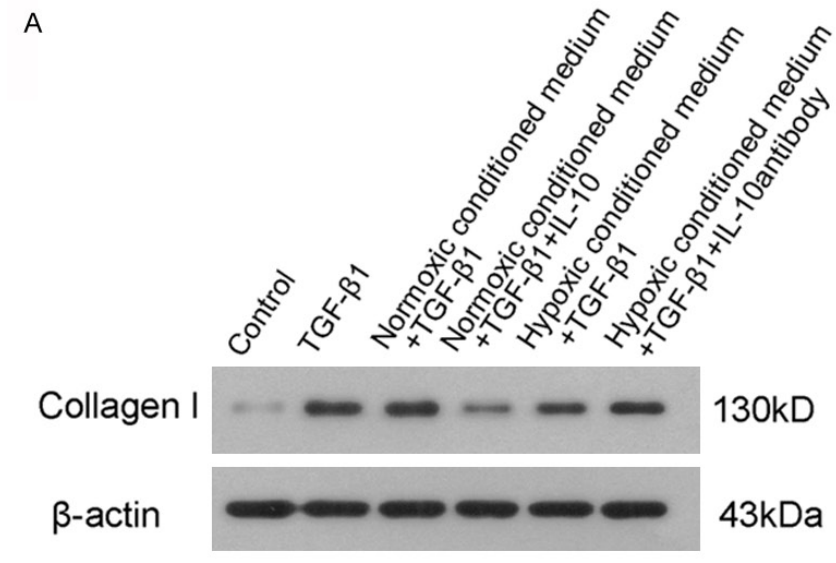
Figure 4. Hypoxic conditioned medium of PMSCs inhibits TGF-β1-induced differentiation of skin fibroblasts to myofibroblast. A. Relative mRNA level of α-SMA was measured using qRT-PCR after treatment with TGF-β1 and normoxic or hypoxic conditioned medium of PMSCs. The mRNA level of α-SMA was normalized to β-actin. B, C. The protein level of α-SMA was detected by western blot and the relative protein level of α-SMA was calculated using β-actin as an internal reference. D. Immunofluorescence was used to detect the expression and distribution of α-SMA. Each experiment was repeated three times and the results are presented as mean ± SD. $P < 0.05$ was considered to be significant.

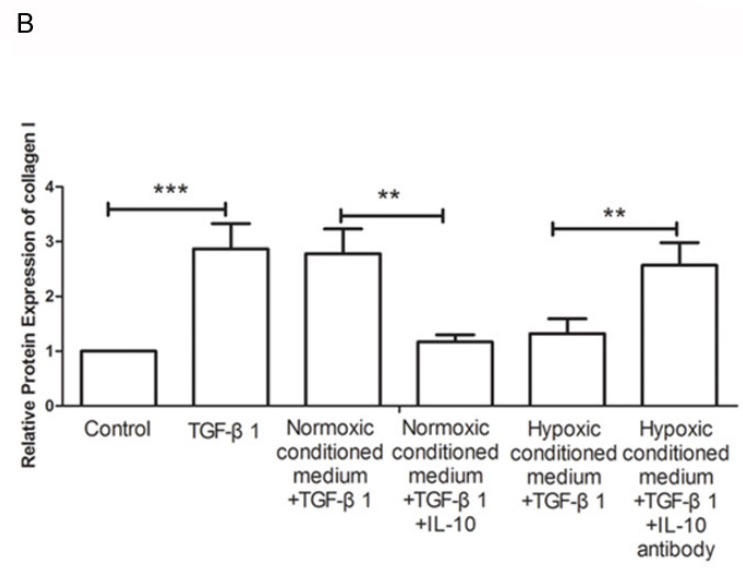
cence also showed that the expression and distribution of α-SMA was changed by TGF-β1, but was reversed by hypoxic conditioned medium of PMSCs; and IL-10 simulated the effects of hypoxic conditioned medium of PMSCs, but IL-10 antibody reversed the effects of hypoxic conditioned medium of PMSCs (**Figure 6**). These results suggest that hypoxic conditioned medium of PMSCs inhibits TGF-β1-induced expression of collagens and differentiation of fibroblasts to myofibroblasts through IL-10.

Discussion

Scars affect the quantity of people's daily lives. In the present study, hypoxic conditioned medium of PMSCs was found to reduce the level of collagens and inhibit the differentiation of fibroblasts to myofibroblasts induced by TGF-β1. Further mechanism study showed that, in hypoxic condition, the level of IL-10 was augmented, treatment with IL-10 simulated the effects of hypoxic conditioned medium of PMSCs and treatment with IL-10 antibody reversed the protective effects of hypoxic conditioned medium of PMSCs. Results of our study suggest that hypoxic conditioned medium of PMSCs performs a protective effect through IL-10.

TGF-β1 is related to the scar formation [2]. In the process of wound healing, the level of TGF-β1 is up-regulated and it drives synthesis of ECM and differentiation of fibroblasts [10]. Lu *et al*. showed that inhibition of TGF-β1 reduced scars [11]. Scar formation is a process characterized by deposition of excessive ECM. In the process of scar formation, fibroblasts


A
Control
TGF-β1
Normoxic conditioned medium +TGF-β1
Normoxic conditioned medium +TGF-β1+IL-10
Hypoxic conditioned medium +TGF-β1
Hypoxic conditioned medium +TGF-β1+IL-10antibody
Collagen I
130kD
β-actin
43kDa


B
Relative Protein Expression of collagen I
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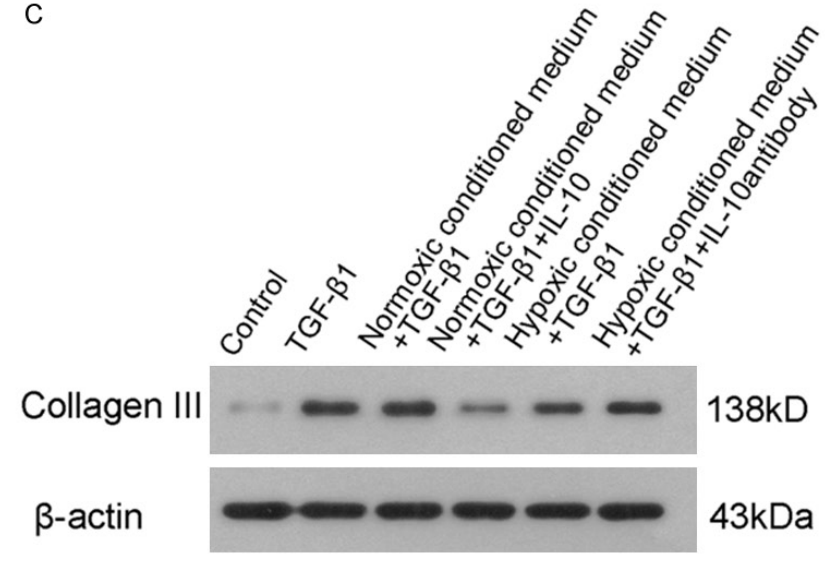
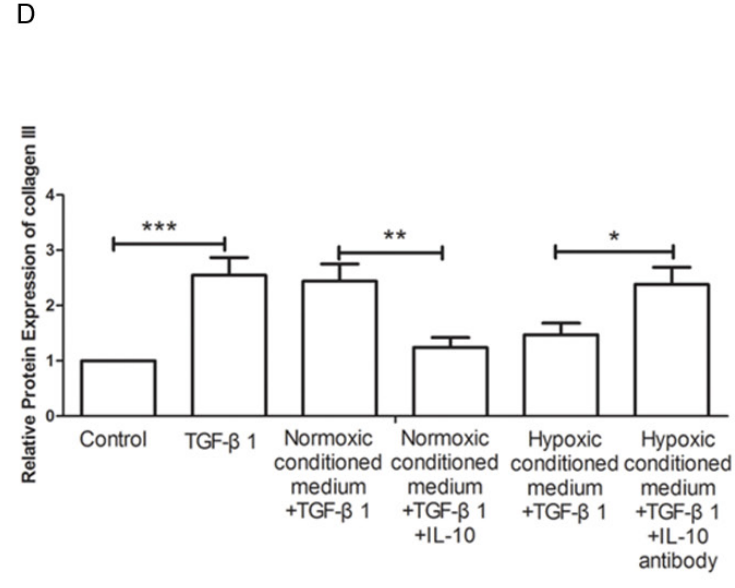
Control
TGF-β 1
Normoxic conditioned medium +TGF-β 1
Normoxic conditioned medium +TGF-β 1 +IL-10
Hypoxic conditioned medium +TGF-β 1
Hypoxic conditioned medium +TGF-β 1 +IL-10 antibody


C
Control
TGF-β1
Normoxic conditioned medium +TGF-β1
Normoxic conditioned medium +TGF-β1+IL-10
Hypoxic conditioned medium +TGF-β1
Hypoxic conditioned medium +TGF-β1+IL-10antibody
Collagen III
138kD
β-actin
43kDa


D
Relative Protein Expression of collagen III
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Control
TGF-β 1
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Hypoxic conditioned medium +TGF-β 1
Hypoxic conditioned medium +TGF-β 1 +IL-10 antibody

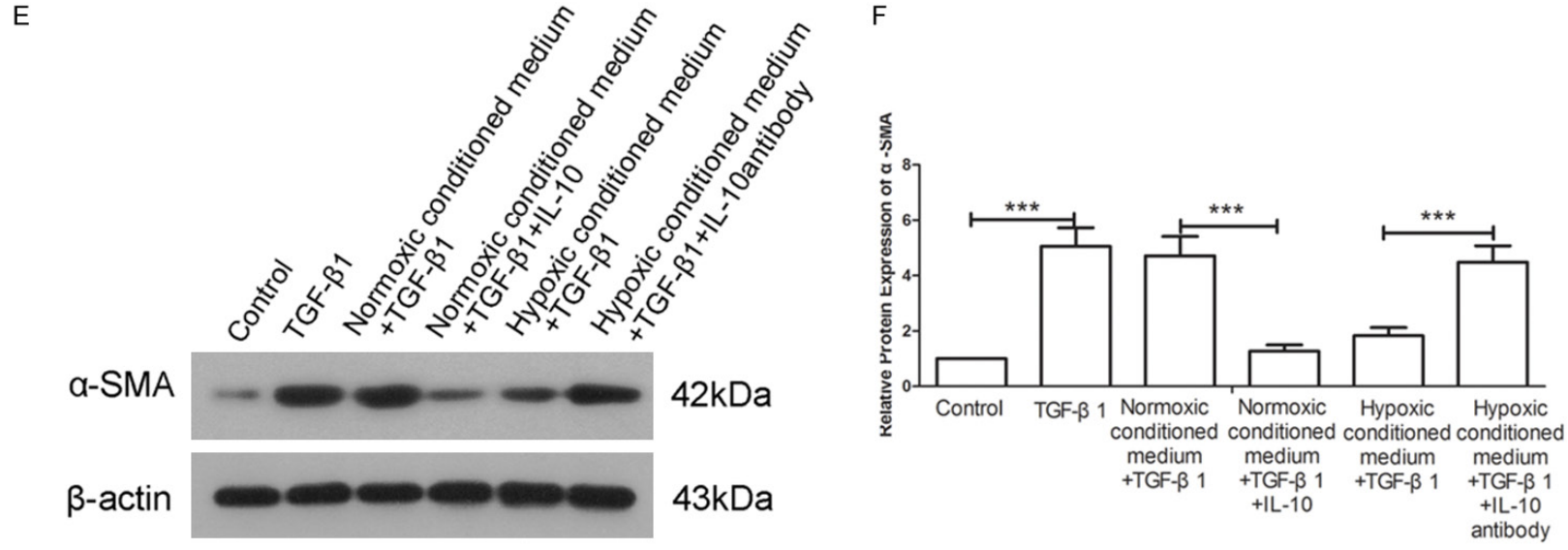

Figure 5. IL-10 is involved in the protective effects of hypoxic conditioned medium of PMSCs. A, B. The protein level of collagen I was detected by western blot after treatment with TGF-β1, normoxic or hypoxic conditioned medium of PMSCs, IL-10 or IL-10 antibody. β-actin was used as the internal reference when the relative protein level of collagen I was calculated. C, D. Collagen III level was detected by western blot with β-actin as the internal reference. E, F. The protein level of α-SMA was detected by western blot after different treatment with β-actin as the internal reference. Each experiment was repeated three times and typical results are presented. The results are shown as mean ± SD. $P < 0.05$ was considered to be significant.

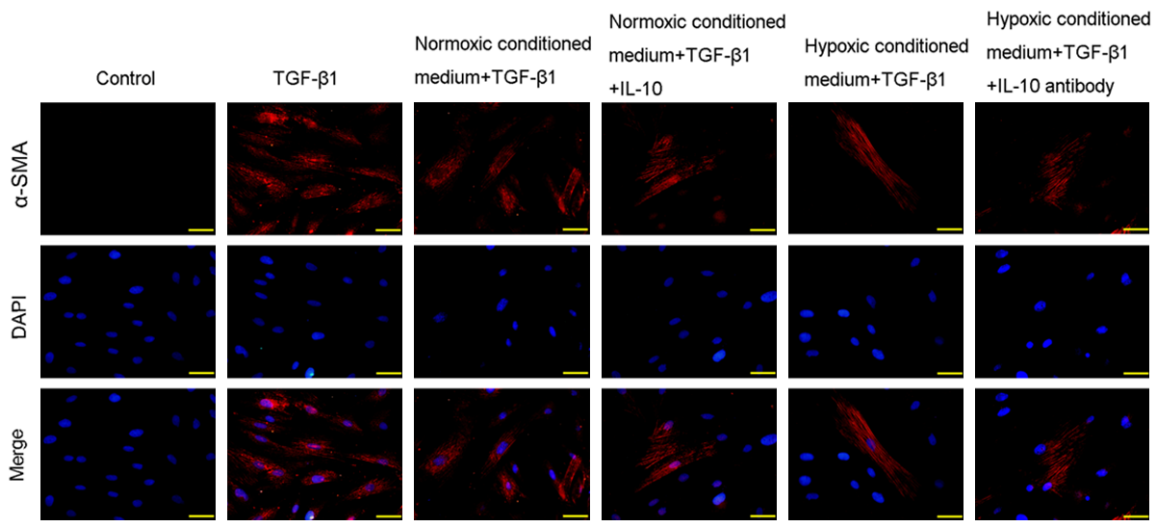

Figure 6. Hypoxic conditioned medium inhibits TGF-β1-induced differentiation through IL-10. Expression and distribution of α-SMA was detected by immunofluorescence. Each experiment was repeated three times and typical results are presented.

are activated, they proliferate and migrate to the wounded areas and synthesized large amounts of ECM, such as collagens and fibronectin, restore the structure of the injured tissues and increase the strength of wounded areas. [7, 10, 12]. In this process, fibroblasts also differentiate to myofibroblasts [13]. In our study, hypoxic conditioned medium of PMSCs was found to decrease the level of collagens and inhibit the differentiation of fibroblasts to myofibroblasts induced by TGF-β1. These results suggest that hypoxic PMSCs perform a protective role in scar formation.

MSCs showed excellently protective function in wound healing [14, 15]. It was reported that MSCs migrated to the injured areas, regulated inflammatory responses, promoted repair and regeneration of damaged tissues through differentiation and paracrine signaling [16]. Paracrine signaling is regarded as the main way for MSCs to perform their protective function [17] and conditioned medium of MSCs also showed protective roles in wound repair in other report [18]. MSCs also inhibit the fibrosis, which is a key factor in scar formation, through paracrine signaling [19, 20]. PMSCs also show a protective role in wound healing [5] and results of our study demonstrated that the conditioned medium of PMSC showed a protective function against scar formation, which provides further evidence for the possible use of PMSCs in wounds treatment.

Hypoxic condition is usually seen in wounded areas [16]. Hypoxia alters biological characteristics of MSCs [21]. Hypoxia was reported to promote the proliferation, differentiation, and metastasis of MSCs [22] and also enhance the protective role of MSCs [23]. Reports showed that hypoxia enhance the protective function of MSCs [18, 23, 24]. Our previous study [8] and results of our study also indicates that hypoxia enhanced the protective role of PMSCs in scar formation.

IL-10 appears as a new therapy for scars [25, 26]. IL-10 contributes to scarless wound healing [27, 28]. IL-10 reduces scar formation via inhibiting the production of collagens and promoting the expression and secretion of MMPs, and inhibiting the differentiation of fibroblasts to myofibroblasts [7, 29]. In our study, we found that hypoxic conditioned medium of PMSCs enhanced expression and secretion of IL-10. These results suggest that the protective role of hypoxic PMSCs may be associated with IL-10. Further study showed that treatment with IL-10 simulated the effects of hypoxic conditioned medium of PMSCs, but inhibition of IL-10 reversed the protective effects of hypoxic conditioned medium of PMSCs. These results provided evidence for our hypothesis that hypoxic PMSCs may perform their protective functions through IL-10. IL-10 also contributes to the regulation of inflammatory responses [30]. Mei *et al.* and Krasnodembskaya *et al.* showed that

PMSCs also show antimicrobial activities which will be beneficial for the clearance of wounds [31, 32], and this antimicrobial activities may also be associated with the regulation of IL-10, but more exploration are still needed.

In the present study, hypoxic conditioned medium of PMSCs was found to inhibit TGF-β1-induced production of ECM and differentiation of fibroblasts through IL-10. Our study suggests that hypoxic PMSCs play a beneficial role in prevention against scar formation and may be used in the treatment of wounds which may result in scarless wound healing.

Acknowledgements

This study was supported by a grant from the Doctoral Scientific Research Foundation of Liaoning Province (NO. 201501003).

Disclosure of conflict of interest

None.

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