Bone marrow mesenchymal stem cells alleviate the formation of pathological scars in rats

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Introduction: Although bone marrow-derived mesenchymal stem cells (BMSCs) have attracted increasing attention because of their pivotal functions in the process of wound healing and fibrosis alleviation, the underlying molecular mechanisms have been poorly understood. Moreover, transforming growth factor beta 1 (TGF-β1) is positively correlated with scar formation, whereas TGF-β3 inhibits the pathological scar formation process. However, the relation of TGF-β1, TGF-β3, and the TGF-β/Smad signaling pathway with BMSCs is unknown and requires further investigation.

Methods: A cell co-culture platform was used to examine the relationship between BMSCs and dermal fibroblasts (DFs). EdU labelling and cell cycle detection were carried out to examine the viability of DF cells. Transwell and wound healing assays were used to test the cell migration of DFs. The expression of TGF-β pathway components and collagens were determined by RT-qPCR and western blotting. A damaged skin rat model was applied to test the effects of BMSC treatment on skin wound healing.

Results: The results showed that BMSC secretion could inhibit the viability and migration of DFs. Moreover, we observed that the TGF-β1-induced expression of TGF-β1, Smad2, Smad3, COLI and COLIII was attenuated upon BMSC treatment in DFs, while the decrease in TGF-β3 expression was enhanced by BMSCs. Furthermore, BMSC treatment accelerated wound healing and attenuated skin collagen deposition in a damaged skin rat model, leading to the mitigation of cell proliferation and enhancement of cell apoptosis. In addition, the expression of alpha-smooth muscle actin (α-SMA), COLI, and COLII was alleviated by BMSC treatment.

Conclusions: Our results indicate that BMSCs can promote wound healing and inhibit skin collagen deposition, which is associated with the TGF-β/Smad signaling pathway.

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Key messages

Skin scar formation is a common adverse consequence of surgical and pathological wounds, and existing scar removal treatments have displayed limited effectiveness. Studies have reported that bone marrow-derived mesenchymal stem cells (BMSCs) can reduce tissue fibrosis, and inhibition of TGF-β1 expression can suppress the formation of skin scars. However, little is known about the relationship between BMSCs and the TGF-β/Smad signaling pathway. In this study, we isolated BMSCs and dermal fibroblasts (DFs) from rats and additionally established a rat model by creating skin wounds on the back of Sprague Dawley (SD) rats. The cells and models were then used to assess the effect of BMSCs on DFs and the relationship between BMSCs and the TGF-β/Smad signaling pathway in inhibiting skin scar formation.

1. Introduction

The skin is a human tissue that is highly susceptible to a variety of environmental damages. Notably, pathological scars are easily formed after skin damage, which often cause various skin
deformities and dysfunctioning [1]. At present, the clinical treatments aimed at preventing pathological scar formation are not as effective as expected, making the treatment of pathological scars an urgent clinical problem to be solved. The main feature of pathological scars is the excessive deposition of extracellular matrix (ECM) components, such as collagen types I and III (COLI and COLIII), in the dermis, and hyperplasia of cells including fibroblasts and myofibroblasts [2–5].

Transforming growth factor-β (TGF-β) signaling is initiated upon the binding of TGF-β ligands to TGF-β receptors I and II, which leads to the phosphorylation of TGF-β receptor I by TGF-β receptor II. Subsequently, the activated TGF-β receptor I recruits and phosphorylates Smad2 and Smad3 proteins, which are called regulatory Smad (R-Smad) proteins [6]. After forming a heteroprotein complex together with the co-Smad protein SMAD4, the Smad complex translocates into the nucleus and regulates the transcription of target genes with the aid of diverse transcription factors in a context-dependent manner [7]. It has been shown that TGF-β/Smad-mediated signaling plays a pivotal role in the production and deposition of ECM [2].

Some evidence has shown that bone marrow-derived mesenchymal stem cells (BMSCs) may serve as therapeutic agents for wound healing and regeneration of tissues, including the skin [8,9]. Isolation and culture of bone marrow-derived mesenchymal stem cells (BMSCs)

2. Materials and methods

2.1. Isolation and culture of bone marrow-derived mesenchymal stem cells (BMSCs)

Bone marrow was isolated from the femur and tibia of 4-week-old male SD rats (weight 160–200 g) [14] and directly cultured at a density of 1 × 10⁶ cells per cm² in a T25 flask containing Dulbecco’s Modified Eagle Medium (DMEM)/F12 (Thermo Fisher, 31331093, Shanghai, China) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher, 26140079, Shanghai, China). After overnight incubation at 37 °C in a 5% CO₂ atmosphere and removal of non-adherent cells, BMSCs were washed twice with phosphate buffered saline (PBS). Subsequently, fresh culture media were added to the flasks to maintain and expand the attached BMSCs. The cells were passaged at a split ratio of 1:2 once they reached 80%–85% confluency, and the media were replenished every two days.

2.2. Isolation and culture of rat dermal fibroblasts (DFs)

The isolation of DFs was initiated by collagenase mediated digesting the skin collected from newborn rats [15]. The resulting cells were filtered through 100-mesh and 200-mesh cell sieves before being centrifuged at 300×g for 5 min. Thereafter, the supernatant was discarded, and the remaining cell pellet was resuspended in DFs complete medium (Procell, CM-R086, Wuhan, China). Subsequently, these cells were plated in a Petri dish precoated with polylysine and statically incubated at 37 °C for 2 h in a 5% CO₂ humidified incubator before refeeding with fresh media. The cells were harvested for the indicated experiments upon reaching confluency, and the media were changed every three days.

2.3. Immunofluorescence staining assay

The cells were seeded on sterile coverslips for 12 h before being fixed with 4% paraformaldehyde diluted in 1 × PBS (pH 7.4) for 15 min. The fixed cells were permeabilized with 0.5% Triton X-100 in 1 × PBS for 20 min and subsequently blocked in 1% goat serum (BOSTER, AR1009, USA) in 1 × PBS for 30 min. Thereafter, appropriate primary antibodies against vimentin (CST, 5741 S, 1:50, rabbit, Shanghai, China) or CD90 (Abcam, Ab225, 1:100, mouse, Shanghai, China) were applied to the indicated samples. After overnight incubation at 4 °C, cells were washed thrice with PBS and directly probed with the fluorophore-conjugated secondary antibody Alexa Fluor Cy3-labeled goat anti-rabbit immunoglobulin G (BOSTER, BA1032, 1:100, USA) or Alexa Fluor Cy3-labeled goat anti-mouse immunoglobulin G (BOSTER, BA1031, 1:100, USA), and DAPI (Beyotime, C1002, Shanghai, China) for 1 h at room temperature. The images were acquired using an inverted fluorescence microscope (magnification, × 200; SP8, Leica) to determine the subcellular localization of vimentin and CD90.

2.4. Immunohistochemistry and immunofluorescence analysis on tissues

First, indicated tissues from rats were fixed and embedded. After sectioning and removing the paraffin, the endogenous peroxidase activity was diminished by treating the slides with 0.3% hydrogen peroxide, followed by the rehydration. Upon antigen retrieval, the slides were cooled down to room temperature and primary antibody against proliferating cell nuclear antigen (PCNA; Abbam, 18,197, Shanghai, China) diluted (1:100) in 1% BSA solution was supplemented to the slides overnight at 4 °C. After probing with 1:200 diluted secondary antibody (DAKO, E0353, Shanghai, China) for 30 min, slides were incubated with Vectastain complex (Vector Laboratories, PK-6100, Shanghai, China). Afterwards, slides were washed and developed using the DAB reagent. Next, slides were counterstained with Mayer hematoxylin (Sigma, MHS80, Shanghai, China) for a few seconds, dehydrated and mounted. The TUNEL staining was carried out by using the Click-iT™ Plus TUNEL Assay kit with Alexa Fluor™ 594 dye (Invitrogen, C10618, Shanghai, China).

2.5. Experimental models

The experiment was carried out by exposing DFs to four different conditions, namely, blank, BMSC, TGF-β1, and BMSC + TGF-β1. DFs were seeded in the lower chamber (Corning; 3397, Shanghai, China) under all the conditions. For the blank group, DMEM/F12 supplemented with 10% FBS was added to the upper chamber, whereas BMSCs were plated in the upper chamber.
for the BMSC group. In addition, TGF-β1 final concentration of 20 ng/mL was added to the DFs seeded in the lower chamber and DMEM/F12 supplemented with 10% FBS was added to the upper chamber for the TGF-β group. TGF-β1 final concentration of 20 ng/mL was added to the DFs seeded in the lower chamber and BMSCs were added to the upper chamber for the BMSC + TGF-β group. All the cells receiving different treatments were incubated in a 37°C, in a 5% CO2 humidified incubator for 48 h before being harvested for downstream analyses.

2.6. Cell viability analysis

DFs exposed to the indicated treatments and vehicle control were seeded into the wells of a 96-well plate at a density of 5000 cells per well. At approximately 48 h post-seeding, the cells were collected for cell viability analysis using a CCK-8 kit (Beyotime, C0037, Shanghai, China) following the manufacturer’s instructions. In brief, 10 μL of CCK-8 were directly added to the media of each sample, and the absorbance at 450 nm was measured after 4 h incubation at 37 °C in a 5% CO2 humidified incubator. Eight replicates were included for each condition.

2.7. 5-Ethynyl-2 deoxyuridine (EdU) incorporation assays

EdU labelling experiments were carried out by a EdU Apollo 488 kit (RiboBio, C10310-1, Guangzhou, China). Briefly, cells were plated in wells of a 24-well plate. After 16 h, cells were supplemented with medium containing EdU (50 μM) and kept in the incubator for another 4 h. Afterwards, cells were fixed, followed by permeabilization using 0.5% Triton X-100 in PBS for 5 min and stained following the manufacturer’s instructions.

2.8. Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from cells or tissues using Trizol (Aidlab, 252250AX, Beijing, China) according to the manufacturer’s protocol, and 1 μg of total RNA was used to obtain the cDNA through reverse transcription using HiScript Reverse Transcriptase (VAZYME, R101-01/02, Nanjing, China). Next, real-time PCR was carried out using the corresponding primers listed in Table 1 and SYBR Green Master Mix (VAZYME, Q111-02, Nanjing, China). Three technical replicates were included in each group. The reaction was initiated with pre-denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s and 60 °C for 30 s. In the final step, a melting curve was drawn to determine the purity of the products.

### Table 1

| Name          | Primer Sequence (5’-3’) |
|---------------|------------------------|
| Rat β-actin   | Forward CACGATGGAGGGCCCGGACTCATC Reverse TAAAACCTCTTATGGCACAACAGT |
| Rat TGF-β1    | Forward TCAGACATTCGGGAAGCAGT Reverse CCAAGGCGCTGTATCCTCCGTC |
| Rat TGF-β3    | Forward ATAGGTGGCAAGAATCTGC Reverse ATGTCTCCATTGGGCTGA |
| Rat Smad2     | Forward CAGTTGTCAAAATCTTCAACA Reverse CTTGTCGCTCCCGTGATATTCT |
| Rat Smad3     | Forward TTTEGCTCACTTCCTCCCAACT Reverse TCCTGCTCAGTCTCCCTC |
| Rat Col I     | Forward TGACTGGAAAGGCGGAGCT Reverse GAATCCATCGTGACTCCTCT |
| Rat Col III   | Forward TTGTGGAATATGGGCAAGCGG Reverse AAAGATCTTCTGGTGGGG |

Fig. 1. Identification of BMSCs and DFs in rat. (A, B) Isolated BMSCs were characterized using CD29 and CD45 antibodies (flow cytometry) (A), and vimentin antibody (immunofluorescence) (B). (C) Isolated BMSCs were characterized using CD29, CD44, CD45, CD90 and CD105 antibodies (flow cytometry). (D) CD90 detection (immunofluorescence) was performed in BMSCs. (E) Representative images of chondrogenic, adipogenic, and osteogenic differentiated BMSCs.
relative expression of mRNA or fold change was calculated based on the $2^{-\Delta \Delta Ct}$ formula.

### 2.9. Western blot assay

The cells or tissues were lysed with RIPA lysis buffer (Beyotime, P0013B, Shanghai, China) supplemented with 1 mM PMSF (Aladdin, P105539, Shanghai, China). The protein concentration of the resulting lysates was determined using a BCA protein concentration determination kit (Beyotime, P0010, Shanghai, China). Cell lysates containing equal amounts of protein were loaded and separated by 10%–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and the resolved proteins were transferred to a polyvinylidene difluoride membrane (Millipore, IPVH00010). Next, the membrane was blocked in 5% non-fat milk dissolved in Tris-buffered saline containing 0.1% Tween 20 (TBST) to block non-specific binding. After incubation at room temperature for at least 1 h, the membranes were incubated with the appropriate primary antibodies against TGF-β1 (Biorbyt, orb11468, 1:1000, rabbit, Shanghai, China), TGF-β3 (Abcam, Ab15537, 1:500, rabbit, Shanghai, China), Smad2 (Affinity, Af6449, 1:1000, rabbit, Shanghai, China), Smad3 (Affinity, A6362, 1:1000, rabbit, Shanghai, China), COL1 (Abcam, Ab34710, 1:2000, rabbit, Shanghai, China), COLIII (Abcam, Ab7778, 1:5000, rabbit, Shanghai, China), and β-actin (BOSTER, BM0627, 1:500, mouse, USA) overnight at 4 °C. Subsequently, the membranes were washed with TBST thrice and incubated with horseradish peroxidase-conjugated secondary antibody (Beyotime, P0026, Shanghai, China) for 1 h. The protein bands were detected using the ECL detection kit (Beyotime, P0018, Shanghai, China).
peroxidase-conjugated secondary antibodies against rabbit immunoglobulin G (BOSTER, BA1054, 1:5000, USA) or mouse immunoglobulin G (BOSTER, BA1051, 1:5000, USA). Finally, the proteins on the membranes were detected using ECL chemiluminescence. β-actin served as an internal reference, and the gray value ratio was calculated as the relative protein expression level.

2.10. In vivo animal experiments

Twenty-four male SD rats with a body weight of approximately 225 g were selected and divided into three groups: control group, model group, and treatment group. Rats in the blank group received no treatment and served as controls. In the model and treatment groups, symmetrical rectangular 3 cm × 0.3 cm incisions were made on one side of the rat back after hair removal and anesthesia with 2.5% pentobarbital. Subsequently, the whole skin layer of the rat, including the muscle membrane, was excised, and a gelatin sponge of 3 cm × 4 mm × 5 mm was inserted into the incision with the surface of 3 cm × 4 mm facing down. After 3 h, each rat was injected with PBS 500 μL under the gelatin sponge in the model group, whereas in the treatment group, each rat was injected with 500 μL PBS containing 1 × 10⁶ cells/mL BMSCs. Wound healing and scar formation were observed every 2 day at 3 days post-operation, and the width of the wound (scar) was recorded. After 14 days of surgery, the rats were sacrificed, and the scars were excised. Hematoxylin and eosin staining was used to determine the degree of collagen deposition in scar areas. All animal experiments were approved by the Institutional Ethics Committee of the First Affiliated Hospital of Zhejiang University and were performed in accordance with the protocol approved by the Animal Care and Use Committee of Zhejiang University.

2.11. Statistical analysis

The SPSS (version 13.0) software package was used for statistical analysis. All data in this study are presented as the mean ± standard deviation. Statistical significance of data was assessed using an unpaired two-tailed t-test. Statistical significance was set at p < 0.05.

3. Results

3.1. Identification and characterization of isolated BMSCs and DFs

BMSCs have valuable characteristics, including, (i) self-renewal, (ii) multilineage differentiation, (iii) anti-inflammatory, and (iv) damaged tissue regeneration-promoting activity [16,17]. Despite their enormous potential and versatility, the role of BMSCs in wound healing and scar formation processes is still poorly defined. Therefore, in this study, BMSCs and DFs were isolated from the femur and tibia and from the skin of rats, respectively. The isolated DFs were positive for CD29 expression, and negative for CD45 expression (Fig. 1A). The characterization of DFs derived from rat skin was carried out using immunofluorescent staining against the fibroblast marker vimentin (Fig. 1B). The overlay histograms showed that the isolated BMSCs were positive for CD29, CD44, CD90, and CD105 expression and negative for CD45 expression, which are mesenchymal stem cells (MSC) cell surface markers (Fig. 1C). The presence of the MSC surface marker CD90 in the isolated BMSCs was further confirmed by immunofluorescence microscopy (Fig. 1D). Additionally, the aforementioned BMSCs retained their ability to differentiate into chondrocytes, adipocytes, and osteoblasts (Fig. 1E). Taken together, these data suggest the successful isolation of BMSCs and DFs.

![Fig. 3. Factors secreted from BMSCs alleviate TGF-β/Smad signaling.](image-url) (A) RT-qPCR detection of mRNA expression of components in TGF-β/Smad signaling and collagen genes. (B, C) Representative Western blot results (B) and quantification (C) of protein expression of components in TGF-β/Smad signaling and collagen proteins.
3.2. Factors secreted from BMSCs mitigate TGF-β1-induced DFs viability and migration

TGF-β, a cytokine that affects cell growth and differentiation, appears to be essential during wound healing and scar formation. To further explore the relationship among BMSCs, DFs, and TGF-β during these processes, we compared the viability of DFs under different conditions measuring the CCK-8 activity. Proliferation of DFs was significantly reduced in the BMSC group compared to that in the blank group. Surprisingly, the increased viability of DFs due to TGF-β treatment was rescued by the addition of BMSCs (Fig. 2A). These data were further confirmed using EdU labeling assay (Fig. 2B). Cell cycle analysis revealed an accumulation of DFs in the G0/G1 phase in the presence of BMSCs (Fig. 2C). Moreover, migration of DFs was also significantly hindered in the groups receiving BMSCs, as suggested by transwell and wound healing assays (Fig. 2C and D). These cumulative data indicate that BMSC-derived secreted factors could prevent the increase in viability and migratory capacity induced by TGF-β in DFs.
3.3. Factors secreted from BMSCs alleviate TGF-β1-induced collagens expression in DFs

A recent study showed that the mRNA levels of TGF-β1 were decreased in the injured dermis of mice upon the addition of factors secreted from BMSCs [18]. To determine whether BMSCs affected the TGF-β1 signaling pathway during scar formation, real-time PCR and western blotting assays were performed on DFs receiving different treatments for detecting mRNA and protein levels of key molecules involved in the TGF-β/Smad signaling pathway, for instance, TGF-β1, TGF-β3, Smad2, Smad3, COLI, and COLIII. Compared with the control group, both mRNA and protein levels of TGF-β1, Smad2, Smad3, COLI, and COLIII were significantly diminished, except for TGF-β3, in the BMSC group (Fig. 3A–C). Notably, a similar trend was observed when comparing the levels of these molecules between the TGF-β1 and BMSC + TGF-β1 groups (Fig. 3A–C). In conclusion, these data suggest that factors secreted from BMSCs could regulate the expression of molecules involved in the TGF-β/Smad pathway and decrease TGF-β1-induced collagen deposition in DFs.

3.4. BMSCs promote wound healing in rat skin

To further investigate the role of BMSCs during the process of wound healing in rat skin, in vivo animal experiments were performed. At three days after generating the scars, the width of the scar in each condition was monitored and recorded for two weeks after surgery. The data revealed that scar width was dramatically

![Diagram](image_url)
reduced upon the addition of BMSCs (Fig. 4A), suggesting that BMSCs might enhance the wound healing process. Moreover, H&E and Masson’s trichrome staining showed that collagen deposition of wound healing tissues was mitigated by BMSC treatment (Fig. 4B and C). Furthermore, immunohistochemistry and immunofluorescence staining showed that changes in the expression of PCNA, a cell proliferation marker, and Tunel staining, a cell apoptosis marker, in the wound healing areas were significantly alleviated by BMSC treatment (Fig. 4D and E). In addition, the expression of α-SMA, COLI, and COLII was greatly upregulated in the rat scar formation model but dramatically diminished in the BMSC treatment group (Fig. 5). Taken together, these in vivo data demonstrate the therapeutic effect of BMSCs on scar formation by enhancing the wound healing process. These results imply that BMSCs can inhibit skin collagen deposition in vivo, which may be related to the TGF-β/Smad signaling pathway.

4. Discussion

As a common adverse effect of surgical and pathological wounds, skin scars or fibrosis mainly result from myofibroblastic hyperplasia and excessive production of ECM [5]. Despite the presence of Skin scar therapies, there has been no intervention that can interfere with the development of skin collagen deposition, and thereby avoid final scar formation [19]. To gain insight into this process, we established a rat skin scar model to examine whether BMSCs have an inhibitory effect on the development of skin collagen deposition. Our results showed that administration of BMSCs promoted wound healing and inhibited scar formation during wound healing.

Moreover, TGF-β, especially TGF-β1, is considered one of the most active fibrotic cytokines. High levels of TGF-β1 act as a robust driver of myofibroblast differentiation and are highly associated with the formation of skin scars [5,20,21]. To further investigate whether BMSC-mediated inhibition of skin collagen deposition is related to the TGF-β1/Smad signaling pathway, BMSCs and DFs were isolated from rat bone marrow skin, respectively, and the latter were treated with TGF-β1 to induce collagen expression in DFs. Co-culture of BMSCs and DFs indicated that factors secreted from BMSCs could impede TGF-β1-induced proliferation of DFs and the expression of key molecules involved in the TGF-β1/Smad pathway, except for TGF-β3. These results indicated that BMSC-mediated inhibition of collagen deposition might result from the decrease in collagen production and fibroblast proliferation. Recent studies have shown that BMSCs can produce anti-fibrotic growth factors and cytokines, including hepatocyte growth factor, IL-10, and adrenomedullin [11–13]. These factors have been shown to alleviate scar formation by downregulating TGF-β1 and collagen expression and upregulating that of matrix metalloproteinase (MMPs), thereby promoting ECM recovery [22]. These findings are in line with our results that the expression of TGF-β1 mRNA and protein was significantly lower in the treatment group than in the model group (Fig. 3A–C), suggesting that BMSCs inhibit the fibrotic process by regulating TGF-β1.

MMP family members play an essential role in altering the local microenvironment in tissue remodeling processes and ECM turnover [23]. BMSCs promote the degradation and remodeling of the ECM by upregulating MMPs [18]. Moreover, HSP47, a collagen-specific intracellular partner, also participates in the modification and assembly of collagen [24]. Furthermore, it has been shown that overexpression of HSP47 promotes collagen accumulation and fibrosis [25]. However, BMSCs can reduce the expression of HSP47. In addition, increased accumulation of myofibroblasts is a key finding in fibrotic dermis, and is thought to be involved in the pathogenesis of fibrotic disease [26]. We found that BMSCs reduced the viability of DFs and inhibited their proliferation. In summary, the application of BMSCs in fibrotic diseases may help to reduce matrix production, proliferation, and differentiation of myofibroblasts.

The present study suggests that subcutaneous transplantation of BMSCs can accelerate wound healing and reduce scar formation, and that these processes are related to the TGF-β1/Smad pathway. However, the molecular mechanisms involved have not been fully elucidated. Jackson et al. showed that MSC treatment may reduce scar formation during wound healing through paracrine function [1], and in agreement with our cell experiments, factors secreted from BMSCs play an important role in inhibiting collagen deposition in DFs. In addition, another study conducted by Yan Wu et al. revealed that MSCs can migrate to damaged dermis and epidermis and attenuate proliferation of DFs [18]. These results indicate that in animals, not only factors secreted from BMSC, but also the cells themselves are involved in the process of alleviating skin scarring and collagen deposition, suggesting that the mechanisms are diverse and complicated. Hence, further investigations are needed to clarify the pathways involved in BMSC-mediated alleviation of skin scarring and collagen deposition.

Declaration of competing interest

All authors declare that they have no competing interests.

Acknowledgements

This work is supported by the project of Nature Foundation of Zhejiang Province (LY20H060008) and Medical Health Science and Technology Project of Zhejiang Provincial Health Commission (2021KY163).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2022.03.004.

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