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Research Article

Knockdown of Tcf3 enhances the wound healing effect of bone marrow mesenchymal stem cells in rats

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The aim of the present study was to explore the wound healing effect of Tcf3 in rat bone marrow mesenchymal stem cells (BMSCs) and their effects on wound healing. Epidermal growth factor (EGF) and fibroblast growth factors (FGFs) were used to induce BMSCs differentiation into epithelial-like cells. Western Blotting analysis and RT-qPCR were performed to assess the expression levels of Tcf3 and the markers of epithelial-like cells, such as Cytokeratin-18 (CK-18), CK-19 and P63. Cell counting kit-8 (CCK-8) and clone formation assay were carried out to detect cell viability. Immunohistochemistry and HE staining were used to assess the level of Tcf3 protein and skin repair degree, respectively. Rat wound healing model was built to evaluate the effects of BMSCs with altered expression of Tcf3 on wound healing. Results showed that EGF and FGFs stimulation increased the expression of CK-18, CK-19 and P63, improved BMSCs viability, but decreased the expression of Tcf3. Knockdown of Tcf3 in BMSCs increased CK-18, CK-19 and P63 expression and improved cell proliferation, as well as accelerated wound healing process. Moreover, inhibition of Wnt/β-catenin signaling weakened the effect of Tcf3 down-regulation on BMSCs proliferation enhancement. And inhibition of Notch1 signaling impeded the epithelial-like cell differentiation of BMSCs induced by Tcf3 down-regulation. Our study reveals that knockdown of Tcf3 enhances the wound healing process of BMSCs in rat, which provides new approach for accelerating skin regeneration.

Introduction

Wound healing progresses normally in the majority of population; however, it is compromised in several diseases, such as diabetes [1], chronic renal failure [2], and arterial or venous insufficiency [3,4]. Impaired wound healing is also a secondary effect of chronic chemotherapeutic regimens, resulting in complications and increased morbidity [5]. Therefore, it is necessary to search methods that promote wound healing process.

Marrow mesenchymal stem cells (SCs), also referred as to stromal multipotent stromal cells, are self-renewing and expandable SCs. Bone marrow mesenchymal SCs (BMSCs) derived from bones are able to differentiate into adipocytes, osteoblasts, and chondrocytes [6]. Due to their properties of self-renewal and pluripotency, BMSCs are widely used for wound healing recently, and serve as the ideal seed cells for tissue engineering and regenerative medicine. Wu et al. [7] revealed that BMSC-treated wounds exhibited significantly accelerated wound closure, with increased re-epithelialization, cellularity, and angiogenesis not only in normal mice but also in diabetic mice, suggesting a direct contribution of BMSCs to cutaneous regeneration. Moreover, BMSC treatment increased the production of collagen and induced more rapid histologic maturation of wounds compared with untreated wounds in the rat [8]. However, how to

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obtain an adequate number of seed cells and maintain their stable differentiation potential still remains challenging.

Tcf proteins (Tcf1, Tcf3, Tcf4, and Lef1 in mammals) are the DNA-binding transcriptional regulators of the canonical Wnt signaling pathway. Through a highly conserved HMG domain and an amino-terminal β-catenin interaction domain, Tcf proteins promote the transcription of their downstream targets when β-catenin accumulates intracellularly [9]. In adult skin, Tcf3 is naturally expressed in the hair follicle bulge, which is thought to be a niche for the multipotent SCs [10]. Nguyen et al. [11] found that Tcf3 existed in embryonic skin progenitors in mice, and the reactivation of Tcf3 increased the expression of genes related to differentiation inhibition in the committed epidermal cells, suggesting that Tcf3 may function in skin SCs to maintain them in an undifferentiated state. Moreover, Pereira et al. [12] found that Tcf3 impaired embryonic SC self-renewal through inhibiting the transcription of Nanog gene. All findings suggest that Tcf3 may restrain wound healing through delaying the differentiation of SCs.

Tcfs/β-catenin signaling is important in progenitor proliferation, but its function on the progression of wound healing induced by BMSCs is unknown. Therefore, in the present study, we aimed to explore the effects of Tcf3 on BMSCs differentiation, determine if Tcf3/β-catenin signaling pathway involved in BMSCs-induced wound healing process. We used epidermal growth factor (EGF) and fibroblast growth factors (FGFs) to induce BMSCs to transdifferentiate into epithelial cells, and recruited siRNAs targeting rat Tcf3 to down-regulate Tcf3 in BMSCs, hoping to find new approach for accelerating skin regeneration.

Materials and methods

Animals

Adult male, Sprague–Dawley rats (supplied by the Shanghai Experimental Animal Center, Chinese Academy of Sciences, Shanghai, China) with an average weight of 250–300 g were housed in the animal care facility. Rats consumed standard rat chow and water. Age-matched animals were used in any experiment. The study was conducted in accordance with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the United National Institutes of Health. All experimental protocols were approved by the Review Committee for the Use of Human or Animal Subjects of Central South University.

Wound healing model

Wound healing rat models were built according to a previous study [8]. Briefly, rats were fasted overnight before the surgery and were anesthetized with intraperitoneal ketamine (60 mg/kg) and xylazine (8 mg/kg). The abdomen was shaved and the skin was cleansed with Betadine solution. The skin was incised and retracted. A 5-cm long midline abdominal fascial incision was made using a scalpel. The incision was immediately closed with a running prolene suture. The skin was closed with an interrupted nylon suture and after recovery, animals were returned to their housing and allowed chow and water.

Preparation of rat BMSCs and identification

The BMSCs were isolated from the marrow cavities of tibias and femora of Sprague–Dawley rat (8 weeks) according to a previous study [13]. Briefly, the marrow was flushed out from the tibias and femora with Dulbecco's modified Eagle's medium-F12 (DMEM-F12) containing 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were centrifuged and suspended in proliferating DMEM-F12 medium containing 10% fetal bovine serum (FBS), 100 mmol/l non-essential amino acids, 100 mmol/l sodium pyruvate, 2 mmol/l L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Then they were placed on a culture dish and cultured in an incubator under humidified atmosphere of 5% CO_2 in air at 37°C. The non-adherent cells were removed on day 3 and the proliferating medium was changed every 2 days. Cells were passaged at 80% confluence. After two or three passages, cells were used in following tests. For BMSCs identification, the third passage BMSCs were harvested and incubated with anti-rat antibodies (BioLegend) conjugated with fluorescein isothiocyanate (FITC)/phycoerythrin (PE)-labeled markers of BMSCs (CD29 and CD90) or hematopoietic cells (CD11b/c and CD45) on ice for 30 min. FITC/PE–conjugated IgG were used as isotype control. After washing three times, cells were analyzed on Fluorescence Activated Cell Sorter (FACS).

Induction of BMSCs differentiation to epidermal-like cells

BMSCs were seeded in 24-well plates at densities of 1–3 $\times$ 10^4 cells/well in 1 ml of medium and were left to attach for 2–3 h. Culture medium for the BMSCs culture systems contained FAD (epidermal stem cell culture medium) and DMEM-F12 (the ratio of FAD:DMEM-F12 is 1:1), 30 ng/ml EGF and 10 ng/ml FGFs. Media were changed twice a week. XAV-939 (20 μM) and DAPT (10 μM) purchased from Cell Signaling Technology (MA, U.S.A.) were used to inhibit Wnt/β-catenin and Notch1 signaling pathways, respectively.

Treatment with BMSCs

The BMSCs harvested between 3 and 5 passages were used in all cell therapy experiments. Local treatment with BMSCs was used in the present study, at the wound site, 6×10^6 BMSCs were resuspended in 0.5 ml of PBS and 50 μl of cell suspension (0.6×10^6 cells) was injected at ten different sites 1 mm lateral to the wound along the entire length of the incision immediately after closing. Operated animals treated with saline served as controls in this experiment.

Immunohistochemical analysis

Corresponding sections of wound tissues from different groups were incubated with the following primary antibodies: anti-Tcf3 (diluted 1:200), (Abcam, MA, U.S.A.). IgG conjugated with peroxidase was used as the secondary antibody (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China). In negative controls, incubation with the above antibodies was omitted. The sections were examined using an upright light microscope (Olympus, Tokyo, Japan). The immunohistochemical staining intensity and area were evaluated by three independent investigators.

Evaluation of wound healing with wound bursting strength

Animals were killed with an overdose of ketamine and xylazine on days 7, 14, and 21, and their skin and abdominal walls were removed for determination of WBS. The suture was removed, and skin with 1 cm width and 4 cm length was removed from the mid-portion of the wound. Then, the skin was placed between the graspers of a DFGS10 force gauge (Chatillon, NY). The tissue was split at 15 mm/min and the highest WBS in Newtons was recorded.

RNA interference and cell transfection

SiRNAs targeting the rat *Tcf3* gene and their controls were purchased from Origene (No. MA, U.S.A.). BMSCs were transfected with siRNAs-Tcf3 and their controls using Lipofectamine 2000 (Invitrogen, WM, U.S.A.) according to the manufacturer's instructions. A total of 2×10^5 cells were transfected with 110 pmoles of siRNA. The transfection effects of siRNA-Tcf3 were detected by Western blotting 48 h after transfection and 24 h for quantitative real-time PCR (qPCR).

Clone formation assay

In the clone formation assay, 2×10^2 BMSCs cells were plated into six-well plates and cultured for 14 days. The colonies were then fixed for 5 min with 10% formaldehyde and stained with 1.0% Crystal Violet for 30 s.

Cell counting kit-8 assay

Cell proliferation was measured using a cell counting kit-8 (CCK-8) assay (Dojindo, Tokyo, Japan). The cells were seeded in 96-well plates in triplicate at densities of 1×10^3 cells per well. Cell proliferation was monitored at different times. After incubation for the designated times, 10 μl of CCK-8 solution were added to each well and incubated for a further 2 h. The absorbance at 450 nm was measured using a microplate reader (Bio-Rad, IQ, U.S.A.).

RNA isolation and quantitative real-time PCR (qPCR)

Total RNA was extracted from the cultured cells with TRIzol (Invitrogen, MA, U.S.A.) reagent, and the contaminated genomic DNA was removed with Deoxyribonuclease I (Invitrogen, MA, U.S.A.). The first-strand complementary cDNA was synthesized using the SuperScript™ III Reverse Transcriptase (Invitrogen, MA, U.S.A.). qPCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, U.S.A.) in combination with a CFX96 system (Bio-Rad, IQ, U.S.A.). The relative mRNA level was expressed as fold change relative to untreated controls after normalization to the expression of GAPDH by the $2^{-\Delta\Delta C}{}_T$ method. The thermocycler parameters were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The target genes and primer sequences are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference.

Western blotting analysis

Total proteins from cells or tissue samples were extracted using RIPA buffer containing protease inhibitor (Beyotime Institute of Biotechnology, China) on ice. For nuclear protein extraction, a CelLytic™ NuCLEAR™ extraction kit (Sigma, Darmstadt, U.S.A.) was used, according to the manufacturer's instructions. The concentration of proteins in the sample was detected by a BCA protein assay kit (Thermo Fisher Scientific, MA, U.S.A.). The proteins were separated by 10% SDS/PAGE and electrotransferred to a PVDF membrane (Merck Millipore, Darmstadt, U.S.A.). The membrane was then blocked in 5% non-fat milk for 1 h and incubated overnight with primary antibodies at 4°C. The

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Table 1 Primer sequences for qPCR analysis

Gene name	Forward sequences	Reverse sequences
CK-18	GGGGCCACTACTTCAAGACC	CATGGCCAGTTCCGTCTCAT
CK-19	TTTGGGTCAGGGGGTGTTTT	GAGGCGATCGTTCAGGTTCT
P63	TGTGTCGGAGGAATGAACCG	TACGTCTCGATCGTGTGCTG
Tcf3	CCAGTGAGATCAAGCGGGAG	CTGTACTGCTGGTACGTGTG
GAPDH	GCGAGATCCCGCTAACATCA	CTCGTGGTTCACACCCATCA

next day, the PVDF membrane was washed with 1× TBST, followed by incubation for 1 h with horseradish peroxidase (HRP)-labeled secondary antibodies diluted with 5% non-fat milk. Finally, the protein bands were detected using an ECL system (GE, U.S.A.). GAPDH and Histone were used as an endogenous control for total and nuclear proteins, respectively. The primary antibodies in the present study included the following: anti-GAPDH (1:5000, Sigma–Aldrich, Darmstadt, U.S.A.), anti-Histone H (1:5000, Abcam), anti-β-catenin (1:1000, Abcam), anti-CK-18 (Cytokeratin-18) (1:1000, Abcam), anti-CK-19 (1:1000, Abcam), anti-P63 (1:2000, Abcam) and anti-Tcf3 (1:2000, Abcam).

Luciferase report of Wnt and Notch1

3′UTR segments of the *Wnt* gene were amplified by PCR from the total cDNA of BMSCs and inserted into the pMIR-REPORT™ Luciferase (pMIR-R-L) control vector (Ambion) using HindIII and SpeI as restriction sites. Target segments and mutant inserts were confirmed by sequencing. *Renilla* luciferase vector was used for normalization. The cells were co-transfected in 24-well plates using Lipofectamine 2000 according to the protocol of the manufacturer with 0.2 μg pMIR-R-L vector and 0.04 μg control vector. pMIR-R-L and *Renilla* luciferase activities were measured consecutively using the dual-luciferase reporter assay system (Promega, MA, U.S.A.) 48 h after transfection.

Dual luciferase assay was used to detect the signal of Notch pathway using a commercial Notch Pathway Reporter kit (BPS Bioscience Corp., San Diego, California), according to specification.

Statistical analysis

Each experiment was performed at least three times. Statistical analyses, including two-tailed tests, unpaired Student's *t* tests, and a one-way analysis of variance, were performed using SPSS 23.0. *P*-values <0.05 were considered as statistical significance.

Results

Tcf3 expression levels negatively correlate with the proliferation and differentiation of BMSCs

First, we used flow cytometry analysis to determine whether BMSCs were successfully isolated. The results revealed that the cells were positive for CD29 and CD90, while negative for the hematopoietic surface markers CD11b/c and CD45 (Figure 1A). Then, we used both FGFs and EGF to induce BMSCs transdifferentiation, As shown in Figure 1B, BMSCs tended to show more epithelial phenotype. Besides, EGF+FGFs treatment significantly increased BMSCs proliferation (Figure 1C) and clone formation ability (Figure 1D). What was more, we detected the expression of epithelial cells markers in the stimulation of EGF+FGFs in BMSCs. Both the mRNA and protein expression levels of CK-18, CK-19, and P63 were elevated when adding EGF+FGFs to the culture medium of BMSCs as compared with the PBS group or bFGF group (Figure 1E–G), but the expression levels of Tcf3 were reduced (Figure 1E–G), indicating that EGF and FGFs could induce BMSCs transdifferentiated to epithelial cells. These results uncovered that Tcf3 expression was negatively correlated with BMSCs transdifferentiation.

Knockdown of Tcf3 accelerates BMSCs proliferation and epithelial-like cell transdifferentiation

Then we explored the function of Tcf3 on BMSCs proliferation and epithelial-like cell transdifferentiation using the siRNAs to down-regulate Tcf3. Figure 2A,B showed the knockdown efficiency of Tcf3 both in mRNA and protein levels, and siRNA-1 and siRNA-3 presented higher knockdown efficiency than siRNA-2. Down-regulation of Tcf3 enhanced cell proliferation (Figure 2C) and clone formation ability (Figure 2D,E) in BMSCs, as well as improved the expression levels of CK-18, CK-19, and P63 (Figure 2F–H). These data suggested that knockdown of Tcf3 enhanced BMSCs proliferation and accelerated epithelial-like cell transdifferentiation.

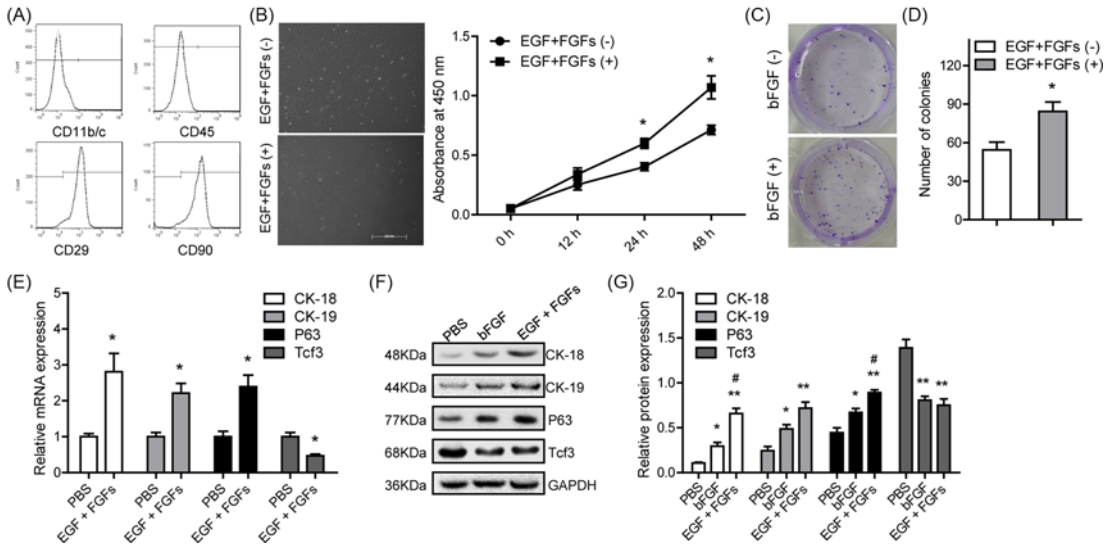

Figure 1. The effects of EGF and FGFs on the proliferation and differentiation of BMSCs

(**A**) FACS was used to detect the expression of CD11b/c, CD45, CD29, and CD90 on the cytomembrane after BMSCs being treated with EGF+FGFs for 48 h. (**B**) The cellular morphology of BMSCs before and after the treatment of EGF+FGFs (scale bar = 200 μm). (**C**) CCK-8 assay was performed to detect the proliferation of BMSCs after treatment with EGF+FGFs for various times (0–48 h). (**D**) Clone formation assay was carried out to determine the clone formation ability of BMSCs after treatment with EGF+FGFs for 48 h. (**E**) The mRNA levels of CK-18, CK-19, P63, and Tcf3 after BMSCs were treated with EGF and FGFs were detected by using qRT-PCR. (**F**,**G**) Western blotting analysis of the protein expression levels of CK-18, CK-19, P63, and Tcf3 after BMSCs treated with bFGF or a combination of EGF and FGFs. The expression of protein was normalized to GAPDH. The data presented are the mean ± standard deviation (SD) and represent three independent experiments. (*$P<0.05$, **$P<0.01$, compared with PBS group; #$P<0.05$, compared with bEGF group; unpaired Student's *t* test for (C–E); one-way analysis for (G)).

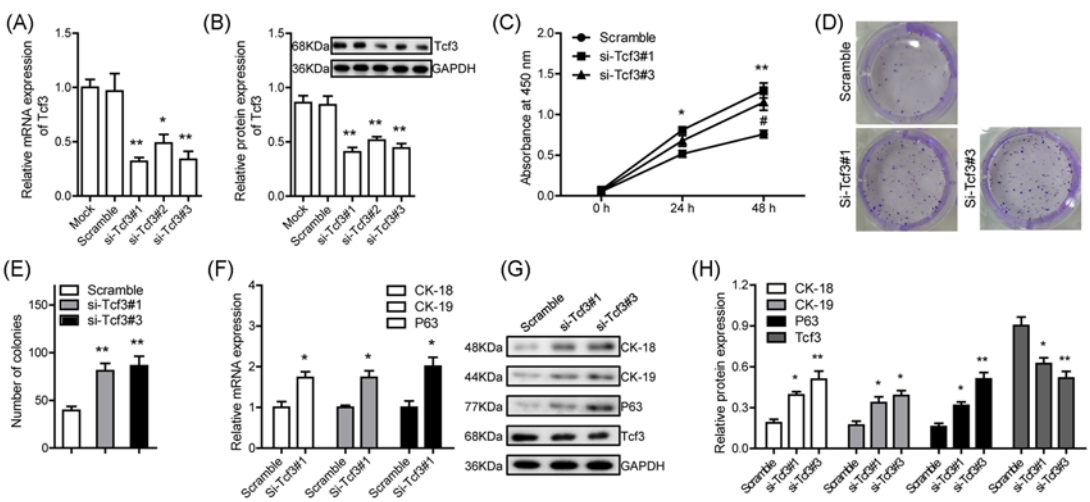

Figure 2. The function of Tcf3 on BMSCs proliferation and differentiation

(**A**,**B**) Tcf3 was knocked down by three specific siRNAs (si-Tcf3#1, si-Tcf3#2, and si-Tcf3#3). (**C**) CCK-8 assay was chosen to evaluate the function of Tcf3 on BMSCs proliferation. (**D**,**E**) Clone formation assay was carried out to determine the proliferation of BMSCs after transfection with siRNA-Tcf3. (**F**–**H**) The mRNA and protein levels of CK-18, CK-19, P63, and Tcf3 after the BMSCs were transfected with siRNA-Tcf3. The expression of protein was normalized to GAPDH. The data presented are the mean ± standard deviation (SD) and represent three independent experiments (*$P<0.05$, **$P<0.01$, unpaired Student's *t* test).

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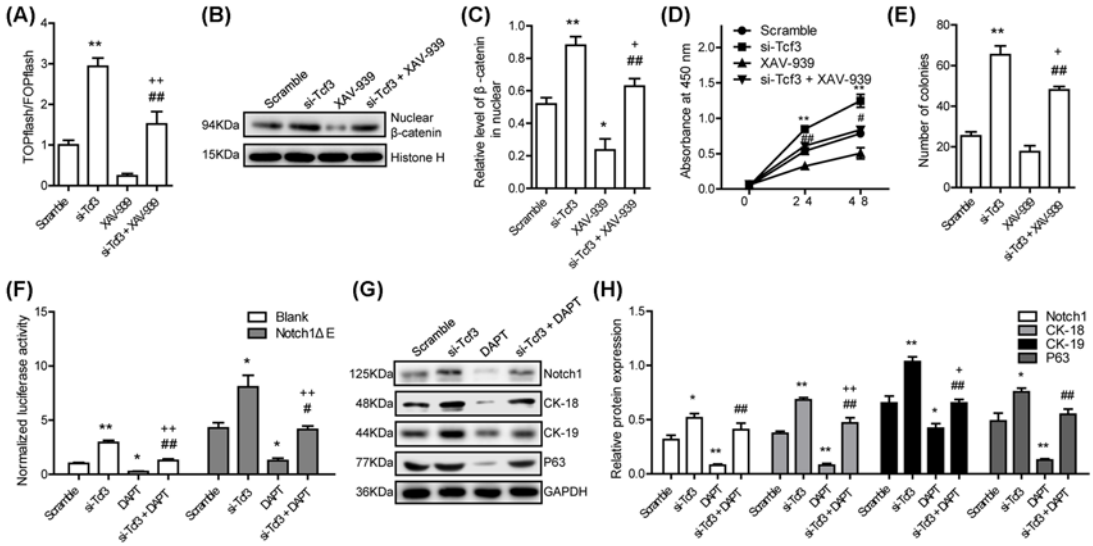

Figure 3. The underlying mechanism of Tcf3 on BMSCs proliferation and differentiation

(**A**) The activity of Wnt was detected using luciferase reporter system (XAV-939 was used to inhibit Wnt/β-catenin signaling). (**B**,**C**) Western blotting was performed to detect the nuclear translocation of β-catenin. (**D**) CCK-8 assay was carried out to measure cell proliferation after various treatments. (**E**) The proliferation of BMSCs was determined using clone formation assay. (**F**) Dual luciferase assay was used to detect the signal of Notch pathway using a commercial Notch Pathway Reporter kit. (**G**,**H**) Western blotting analysis of CK-18, CK-19, P63, and Tcf3 expression in BMSCs after different treatments. The expression of the nuclear protein was normalized to that of Histone, and the expression of the total protein was normalized to that of GAPDH. The data presented are the mean ± standard deviation (SD) and represent three independent experiments ($^{*}P<0.05$, $^{**}P<0.01$, si-Tcf3 v.s. Scramble, $^{\#}P<0.05$, $^{\#\#}P<0.01$, si-Tcf3 + XAV-939 vs. si-Tcf3, $^{+}P<0.05$, $^{++}P<0.01$, si-Tcf3 + XAV-939 vs. XAV-939, one-way analysis).

Knockdown of Tcf3 accelerates BMSCs proliferation through activating Wnt/β-catenin and promotes epithelial-like cell transdifferentiation through Notch1 signaling

To explore the molecular mechanisms of Tcf3 on BMSCs proliferation and transdifferentiation, we explored if Wnt/β-catenin and Notch1 signaling pathways were involved. XAV-939 and DAPT were used to inhibit Wnt/β-catenin and Notch1 signaling pathways, respectively. The activity of Wnt was detected using luciferase reporter system, as Figure 3A displayed, Wnt activity was strengthened when knockdown Tcf3 in BMSCs, while Wnt activity was cut down when adding XAV-939. Also, results from the Western blotting analysis showed that Tcf3 down-regulation increased the nuclear expression of β-catenin, while XAV-939 showed the opposite result (Figure 3B,C), suggesting that Tcf3 could negatively regulate the activity of Wnt/β-catenin signaling. Besides, compared with the siRNA-Tcf3 group, cell clone formation and cell growth abilities were all decreased when adding XAV-939 to the culture medium of BMSCs on the basis of siRNA-Tcf3 (Figure 3D,E). These results demonstrated that Wnt/β-catenin signaling was necessary for siRNA-Tcf3 induction of BMSCs proliferation.

Moreover, dual luciferase assay showed that the activity of Notch1 pathway was increased when knockdown of Tcf3 in BMSCs, but its activity and expression level were decreased when DAPT was added (Figure 3F). Besides, DAPT weakened the effect of Tcf3 down-regulation on the promotion of CK-18, CK-19, and P63 expression (Figure 3G,H). These data illustrated that knockdown of Tcf3 promoted epithelial-like cell transdifferentiation of BMSCs through activating Notch1 signaling.

Knockdown of Tcf3 promotes the wound healing effect of BMSCs on wounded skin of rat models

Finally, we explored the role of Tcf3 on the wound healing ability of BMSCs *in vivo*. Tcf3 protein and mRNA expression levels were elevated significantly in the wounded skin tissues of saline rats, in a time-dependent manner (Figure 4A–C). Tcf3 expression was decreased in the wounded skin tissues of BMSCs-treated mice, especially for si-Tcf3

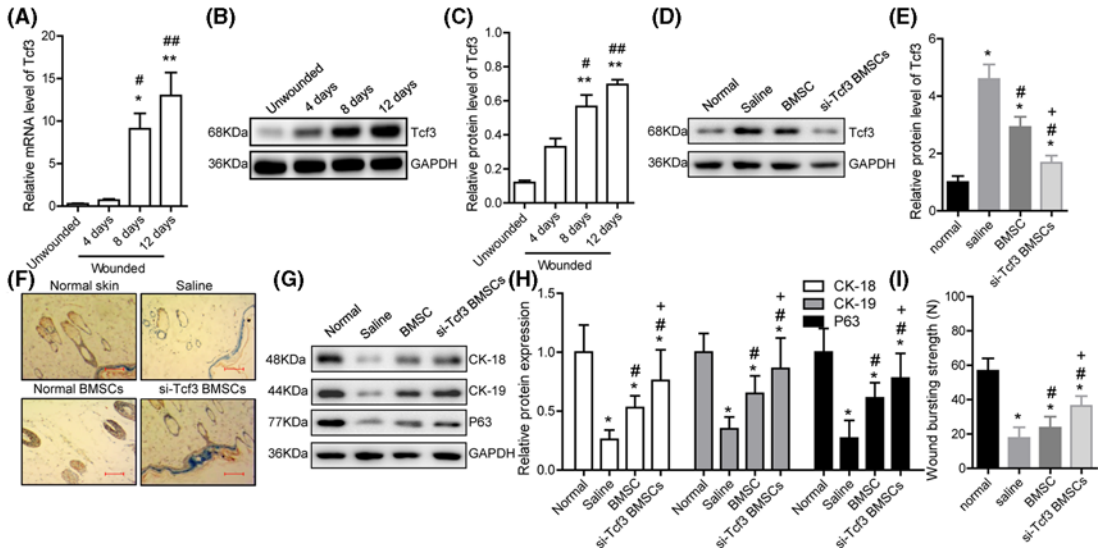

Figure 4. Tcf3 knockdown enhanced the effect of BMSCs on wound healing improvement

(**A–C**) qRT-PCR and Western blotting analysis were carried out to assess the mRNA and protein levels of Tcf3 in the wounded skin tissues after 0, 4, 8, and 12 days of injury in rats from the saline group. (**D**,**E**) Western blotting analysis of the protein expression of Tcf3 in the skin tissues of rats from different groups after 12 days of the treatments. (**F**) Giving the impaired skin different treatment, such as saline, normal BMSCs and siRNA-Tcf3 BMSCs, after 12 days of treatments, immunohistochemical analysis was performed to assess Tcf3 protein level (Scale bar = 100 μm). (**G**,**H**) Western blotting analysis of the expression of CK-18, CK-19, and P63 in the skin tissues of rats from different groups after 12 days of the treatments. (**I**) Wound bursting strength was used to evaluate the wound healing of rats with different treatments. The expression of the total protein was normalized to that of GAPDH. The data presented are the mean ± standard deviation (SD) and represent three independent experiments (*P<0.05, **P<0.01, compared with normal group; $^{\#}P$<0.05,$^{\#\#}P$<0.01, compared with saline group; ^{+}P<0.05, compared with BMSCs group; one-way analysis).

BMSC-treated group as compared with the saline group (Figure 4D–F). Besides, si-Tcf3 BMSCs treatment significantly enhanced the expression of CK-18, CK-19, and P63 in the wounded skin tissues as compared with BMSC group (Figure 4G,H). Moreover, the wounded skin showed higher wound bursting strength in si-Tcf3 BMSCs group than BMSC group (Figure 4I). Overall, these findings indicated that Tcf3 down-regulation enhanced the function of BMSCs on wound healing.

Discussion

BMSCs therapy is a novel, therapeutic option for the acceleration of skin healing, according to their character to differentiate into a variety of tissue types *in vitro*. In our study, we focused on the effects of Tcf3 on the epithelial-like cell transdifferentiation of BMSCs, aiming to find potent method to enhance BMSCs function on wound healing. And our results revealed that knockdown of Tcf3 facilitated the epithelial-like cell transdifferentiation of BMSCs, which then promoted the effects of BMSCs on accelerating wound healing process.

In our study, we used EGF and FGFs to induce BMSCs differentiate into epithelial-like cells which have the potential to enhance wound repair [14–16]. We found that EGF and FGFs treatment promoted the expression of CK-18 [17], CK-19 [18], and P63 [15] which are always stably expressed in normal epithelial tissues, and their positive expression can be proven as epithelium derived. Additionally, we observed that Tcf3 expression level was negatively correlated with the expression levels of CK-18, CK-19, and P63, indicating that Tcf3 might exert an opposite effect from EGF and FGFs and protect BMSCs from transdifferentiation into epithelial-like cells. Similarly, Nguyen et al. [11] revealed that Tcf3 expression was required to keep the undifferentiated state of skin SCs, while reactivation of Tcf3 led to the inhibition of Wnt signaling and the expression of genes related to cell differentiation in stereotyped skin epidermal cells, suggesting that Tcf3 down-regulation would be beneficial for SC differentiation. Moreover, we showed that knockdown of Tcf3 promoted cell proliferation, clone formation and the expression of CK-18, CK-19, and P63 in BMSCs, uncovering a novel role for Tcf3 in BMSCs viability.

We also built the *in vivo* rat wound healing models to explore the effects of Tcf3 in the wound healing process induced by BMSCs. We demonstrated that Tcf3 expression was increased in the wound skin tissues in a time-dependent

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way, which was consistent with previous study [19]. Besides, we found that knockdown of Tcf3 significantly increased the effects of BMSCs on wound healing promotion, with elevated expression of CK-18, CK-19, and P63 and enhanced wound bursting strength. However, Miao et al. [19] showed that Tcf3 up-regulation promoted the migration of keratinocyte *in vitro* and contributed to the improvement of wound healing *in vivo*. I think that the seemingly inconsistent results might be caused by the different cell types and experimental methods. Miao et al. [19] aimed to explore the role of Tcf3 on keratinocyte migration and wound healing through recruiting the direct Tcf3-transgenic mice, while the current study aimed to probe Tcf3 effects on the epithelial-like cell transdifferentiation of BMSCs and then explore their function in wound healing.

Moreover, we observed that knockdown of Tcf3 accelerated BMSCs proliferation through activating Wnt/β-catenin signaling. Tcf3 belongs to the Lef/Tcf family of transcription factors, all of which contain a highly conserved HMG domain that binds to a conserved recognition sequence and domains that interact with the transcriptional activator β-catenin and Groucho/TLE-family corepressors. Lef/Tcf members function as repressors when bound to Groucho/TLE corepressors or as activators when bound to β-catenin [20,21]. In most cases, nuclear β-catenin interacts with Lef/Tcf members to activate transcription, converting them from corepressors into coactivators [19]. Miao et al. [19] revealed that Tcf3 could promote keratinocyte migration without its β-catenin-interacting domain, which presented a novel β-catenin-independent role for Tcf3 and underlined the importance of Tcf3's ability to act as a transcriptional repressor. However, our results demonstrated that down-regulation of Tcf3 enhanced the nuclear accumulation of β-catenin, suggesting that there might be a negative feedback between Tcf3 and β-catenin, which needs to be clarified in our further study.

Moreover, our findings revealed that knockdown of Tcf3 promoted epithelial-like cell transdifferentiation through Notch1 signaling. Notch intracellular domain will be released and translocate to the nucleus when Notch proteins (Notch 1–4) bind to their ligands, Jagged 1 and 2 and Δ-like-1, 3, and 4, driving the expression of Hes1, c-Myc, and other genes that promote cell growth [22] and differentiation [23]. For example, Guo et al. [24] demonstrated that lenalidomide could restore the osteogenic differentiation of BMSCs derived from multiple myeloma patients via deactivating Notch signaling pathway. Notch1 was also found to be positively associated with the differentiation of human BMSCs to cardiomyocytes [25], as well as rat BMSCs to endothelial cells [26]. And our study, for the first time, clarified that Tcf3 down-regulation promoted BMSCs transdifferentiation into epithelial-like cell through activating Notch1 signaling.

It is known that MSCs secrete soluble factors to exert influence over surrounding tissues [27], therefore, the beneficial effects of BMSCs are believed to come from the potent paracrine factors secreted by MSCs, including both anti-inflammatory and immunomodulatory factors, such as the constitutive surface expression of major histocompatibility complex (MHC) class I and variable surface expression of MHC class II [28–30]. MHC class II proteins are potent alloantigens recognized by alloreactive $CD4^+$ T cells and promote T-cell proliferation [31]. Furthermore, Sherman et al. [32] reported that BMSCs would promote wound healing through up-regulation of transforming growth factor (TGF)-β1. And the effects of Tcf3 on the inflammatory reactions in BMSCs need to be further investigated in the present study.

In conclusion, the current study demonstrated that Tcf3 down-regulation enhanced the effects of BMSCs on accelerating wound healing *in vivo*. *In vitro* studies are warranted to allow us to explore the underlying mechanism of Tcf3 on wound healing, indicating that knockdown of Tcf3 increased BMSCs proliferation through activating Wnt/β-catenin and promoted epithelial-like cell transdifferentiation through Notch1 signaling, which benefit in wound healing. Collectively, our study investigated the underlying mechanism of Tcf3-BMSCs on wound healing deeply, which provides new opinions for skin wound healing treatment.

Author Contribution

Bin He participated in almost of the experiments and data analysis, and wrote the manuscript. Jia Chen and Liang Liu did parts of the experiments and data analysis. Hao Wang did parts of the experiments. Shaohua Wang and Ping Li visualized and validated the data. Jianda Zhou provided the idea and revised the writing.

Competing Interests

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Abbreviations

BMSC, bone marrow mesenchymal stem cell; CCK-8, cell counting kit-8; CK-18, Cytokeratin-18; DMEM-F12, Dulbecco's modified Eagle's medium-F12; EGF, epidermal growth factor; FBS, fetal bovine serum; FGF, fibroblast growth factor; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MHC, major histocompatibility complex; PE, phycoerythrin; SC, stem cell.

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