GDF-5 promotes Epidermal Stem Cells proliferation via Foxg1-cyclin D1 signaling

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Research

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Abstract

Objective: Epidermal stem cells (EpSCs) can self-renew and are responsible for the long-term maintenance of the skin, it also plays a critical role in wound re-epithelization, but the mechanism underlying EpSCs proliferation is unclear. Here, we studied the effects of GDF-5 on mouse EpSCs proliferation mechanism in wound healing.

Methods: Firstly, the effects of GDF-5 on EpSCs proliferation was tested by using CCK8 reagent and PCNA expression was analysed by western blotting. Secondly, we screened genes that promote EpSCs proliferation in the FOX family and cyclin by qPCR, and further analysed the protein expression level of the selected genes by Western blotting. Thirdly, siRNA plasmids and pAdEasy adenovirus were transfected or infected, respectively, into mouse EpSCs to detect the effect of target genes on GDF-5 induced cell proliferation. Furthermore, a deep partial thickness burn mouse model was used in which GDF-5 induced EpSCs proliferation was detected by immunohistochemical. Finally, the relationships target genes were analysed by qPCR, immunoblotting and dual luciferase reporter gene detection.

Results: We discovered that 100 ng/ml recombinant mouse GDF-5 was the optimal concentration for promoting mouse EpSCs proliferation. Through preliminary screening by qPCR, we found that Foxg1 and cyclin D1 could be the downstream molecules of GDF-5, and the results were confirmed by Western blotting. And the effect of GDF-5 on mouse EpSCs proliferation was regulated by Foxg1/cyclin D1 in vitro and in vivo. Besides, GDF-5 induced transcription of cyclin D1 was regulated by Foxg1-mediated cyclin D1 promoter activity.

Conclusion: This paper showed that GDF-5 promotes mouse EpSCs proliferation via Foxg1-cyclin D1 signal pathway. It is suggested that GDF-5 may be a new approach to yield EpSCs for wound healing.

Introduction

The epidermis is derived from ectoderm cells during embryonic development, these cells undergo a layering process to form basal, spinous and granular layers [1]. The skin epidermis contains different appendages including sweat glands, hair follicles and sebaceous glands, which are responsible for immune regulation, pigmentation and sensory function [2]. The self-renewal and damage repairing of skin tissue mainly depend on the compensatory proliferation and differentiation of EpSCs [3]. After Billingham and Reynolds first reported skin cell transplantation for wound healing in 1952 [4], EpSCs have been used in clinical practice, repair of burns, acute trauma, skin damage caused by certain diseases [5–7]. But the expansion of EpSCs had always been choke point in its clinical application. The physiological state of EpSCs is affected by different signaling pathways, including MAPK (mitogen-activated protein kinase) [8, 9], Wnt (wingless) [10, 11] and TGF-β (transforming growth factor-beta) [12] signaling, etc. BMP (bone morphogenetic protein) belongs to the TGF-β family and can stimulate cell proliferation [13].
Extracellular BMP binds to cell membrane receptors to initiate downstream signaling pathways, and the signal molecule is translocated to nucleus, where it is combined with a nuclear transcription factor to regulate gene expression [12]. According to report, BMP-4 supports self-renewal by inhibiting MAPK pathways in mouse embryonic stem cells [14]. Growth/differentiation factor 5 (GDF-5) is a BMP family member [15], also known as CDMP-1 and BMP-14. Studies suggest that GDF-5 affects angiogenesis [16], migration [16], apoptosis [17] and differentiation [18] in vitro. Syed H.E and colleagues also discovered that GDF5-induced p38-MAPK signaling in fibroblasts regulate cardiac repairing after myocardial infarction [15]. The predecessors conducted a preliminary study effect of GDF-5 on the wound repair [13], so we speculate that the promotion of GDF-5 may promote the proliferation of EpSCs, but the specific proliferation mechanism of EpSCs promoted by GDF-5 has not been reported. Recent studies have found that FOX (Fork head box) and cyclin are involved in the promotion of cell proliferation by BMP [19].

FOX is a kind of nuclear transcription factors family. The activity of FOX protein can be regulated by phosphorylation, acetylation and protease hydrolysis [20]. It is known that PI3K-AKT/PKB (phosphoinositide-3-kinase–protein kinase B/Akt), TGFβ-Smad and MAPK signaling pathways can affect the level of FOX family proteins [19]. Foxa1, Foxc1, Foxd3, Foxo3, Foxg1, Foxp1 and Foxm1 are associated with cell proliferation [21]. The Foxg1 gene is a dose-sensitive gene, it can antagonize the pro-apoptotic effect of Foxo3 [22] and promotes hepatocellular carcinoma epithelial-mesenchymal transition [23]. As Shasha Zhang's reported, knocking out the Foxg1 gene will increase differentiation of newborn mouse cells [24]. Studies have found that cyclin D plays an important role in cell proliferation, which has three subfamilies: cyclin D1, cyclin D2 and cyclin D3. It mainly initiates signal cascade after binding and activating CDK (4 or 6) (cyclin-dependent kinase 4 or 6) which promoting cell proliferation [25, 26]. Julie A. Siegenthaler reported that Foxg1 was associated with cyclin in promoting intermediate progenitor cell proliferation [27]. Besides, the Wnt/cyclin D1 pathway has a dedifferentiating effect for differentiated epidermal cells [28]. In our previous research, we found that cyclin D1 is an important downstream signaling molecule in the proliferation of EpSCs [29].

Thus, we sought to elucidate mechanism of GDF-5 promotes the proliferation of mouse EpSCs in wound healing. Our results provide evidence that elevated Foxg1 and cyclin D1 expression is associated with GDF-5. After silencing these genes, the promotion effect was weakened. It was verified the same results in vivo. Together, these findings indicated that GDF-5 can be used as a new target to promote EpSCs proliferation.

**Methods And Reagents**

**Animals.**

Both male and female C57BL/6 mice were used in this study. They were obtained from the Experimental Animal Department of the Army Military Medical University, China. All animal procedures were approved by the Committee on the ethics of Animal Experiments of the Third Military Medical University and were
conducted in accordance with the guidelines of the Experimental Animal Department of the Army Military Medical University.

**Preparation of mouse primary EpSCs**

The preparation of primary EpSCs from newborn mice (0-2 days) were described in our previous studies [30]. Firstly, the neonatal mice were sacrificed with a carbon dioxide anesthesia machine. Secondly, soaked in 75% ethanol for 1 min. Thirdly, the back skin was separated with sterile surgical instruments and incubated with 0.5% dispase II (Gibco, #17105041) overnight at 4°C. Next, the epidermis was washed three times with sterile PBS and separated carefully and dissociated with 0.25% Trypsin (Gibco, #25200056) at 37°C for 10 min, the single-cell suspension was passed through 70μm filter (BD Falcon #352350) into a sterile 15 ml tube. Then, the cell suspension was centrifuged at 1000 rpm for 5 min, removed supernatant and resuspend cells in K-SFM (Gibco, 10744019) supplemented with 0.2 ng/ml recombinant mouse EGF (Stem cell, #78016), 100 ng/ml Cholera toxin, 30 mg/ml BPE (Bovine pituitary extract), 0.05 mM Calcium chloride and 100 U/ml of streptomycin and penicillin. Finally, the cells were cultured and the medium was changed every 2-3 days.

**Flow cytometry analysis**

When cells confluence become ~70% after TrypLE™ select (Gibco, #12563029) passaged. Flow cytometry analysis of the purity of passaged EpSCs: Collect EpSCs at a density of 10^6 cell/ml, and then add antibodies, Santa SC23372-CD71-PE 5μl/EP tube, Santa SC19622-CD49f-FITC 5μl/EPT tube, test after 60 minutes incubation at 4°C.

**Cell proliferation assay**

Possible proliferation was assessed by cell viability using Cell Counting Kit-8 (CCK-8) (Beyotime, C0038, China) according to manufacturer’s instructions. The 5,000 EpSCs /well were seeded in 96-well plates, and treated with 0, 1, 50, 100, 500 and 1,000 ng/ml of GDF-5 (Beyotime, P6193, China) for 12, 24, 48, 72 hours. After that, 10 μl CCK-8 solution was added to 96-well and incubated for 2-4 hours. Absorbance was measured at 450 nm with a microplate reader (Spectra Max 190; Molecular Devices).

**Adenovirus infection and siRNA transient transfection**

Adenovirus transfection and siRNA interference protocol was as previously described [31, 32]. Adenovirus transfection was made when EpSCs reach 70% confluence, then aspirated the medium and added fresh medium. After that, added 10 μl Myc adenovirus and 10 μl Foxg1 adenovirus and 10 μl empty vector to each group. After 24 hours’ transfection, we observed the fluorescence intensity and expression ratio with a fluorescence microscope, and changed the medium. After 48 hours’ transfection, cells were collected for subsequent experiments. Specific cyclin D1 siRNA and control siRNA were purchased from Thermo Fisher Scientific. EpSCs preparation method is as described above. Mouse EpSCs were transfected with siRNA
according to the manufacturer's instructions. The efficiency of siRNA interference was analyzed by the following Western blotting.

**Western blotting (WB)**

The levels of C Foxg1, cyclin D1, PCNA and GAPDH protein were detected by WB. First, EpSCs protein samples were prepared using RIPA lysis buffer (Beyotime, P0013B, China), which contained protease and phosphatase inhibitors. It was quantified using BCA protein evaluation kit (Beyotime, P0012S, China). Next, 30 μg/each sample of protein was loaded onto 10% SDS-PAGE and transferred to PVDF membrane (Beyotime, FFP24, China). Then, the membrane was blocked with a 5% milk solution at room temperature for 2 hours. Then, the primary antibody was incubated overnight at 4°C. The primary antibody was diluted according to the following ratio: PCNA (ab92552, 1: 5000), Foxg1 (ab196868, 1: 5000), cyclin D1 (ab16663, 1: 5000) and GAPDH (ab181606, 1: 10000). All antibodies were purchased from Abcam (Cambridge, Massachusetts, USA). After incubating the primary antibody, the membrane was washed three times and incubated with goat anti-rabbit IgG (H+L) (1: 10000) (abcam, ab6702) for 1 hour. The bands were visualized by using the BeyoECL Plus (Beyotime, P0018M, China), and the bands were detected using Image Quant LAS 4000s (GE, USA) [[33]].

**Real-time quantitative PCR (qPCR)**

We used RNAiso Plus (Takara, # 9109) to extract RNA following the instructions. The first step is to remove the genomic DNA (42°C, 2 min; 4°C hold), the second step is the reverse transcription reaction (37°C, 15 min; 85°C, 5 sec; 4°C hold), and finally the Real Time PCR reaction (95°C, 30 sec; go to 39 (40 cycles), 95°C, 5 sec, 60°C, 30 sec; melt curve)). The primerscript RT reagent kit with gDNA Eraser (Takara, # RR047A) and TB green Premix Ex Taq (Takara, # RR820A) were used. Real-Time PCR analysis of mouse cDNA was performed using the 7500 qPCR System (Applied Biosystems). GAPDH serves as an internal reference. Primers synthesized by Sangon Biotech (Shanghai), and primer sequences are listed in Table1.

**Table 1. Primers for the RT-qPCR**
| Primer name | Sequence (5′ to 3′) | Length | Tm (°C) |
|-------------|---------------------|--------|---------|
| GAPDH-F     | GGTTGTCTCCTGCGACTTCA| 20     | 57.5    |
| GAPDH-R     | TGGTCCAGGGTTTTCTTACTCC| 21     | 56.5    |
| Foxa1-F     | TTACAAGGATGCCTCTCCA | 19     | 52.6    |
| Foxa1-R     | TGGCTCTCTGAAAAGCAAG | 19     | 52.4    |
| Foxc1-F     | GGATCGGCTTGAACAACT  | 18     | 52.4    |
| Foxc1-R     | AGAGTGCCCGGAATAGG   | 17     | 54.0    |
| Foxd3-F     | CGTAGAGAAGCGTGGAGGA | 19     | 56.6    |
| Foxd3-R     | GGCAAAGGAGGTGTGAGTG | 19     | 56.5    |
| Foxg1-F     | AACGGGGCTGAGTGTGGA  | 17     | 58.8    |
| Foxg1-R     | CAGGGGTTGAGGGAGTAGG | 19     | 57.6    |
| Foxo3-F     | GAGGATTCGGCCATGCT   | 17     | 55.4    |
| Foxo3-R     | TTCCTTGGTTGCCCAGAG | 18     | 55.1    |
| Foxp1-F     | TCGCCTGGAGGATAGAA   | 17     | 53.4    |
| Foxp1-R     | ATGCAGGTTGGTCATCA   | 17     | 53.5    |
| Cyclin C-F  | ATGCTTGTAATTTGATTTGCT| 21     | 49.5    |
| Cyclin C-R  | CAGGGGTTGAGGGAGTAGG | 19     | 57.6    |
| Cyclin D1-F | ACCCTGACACCAATCTCCT | 19     | 55.6    |
| Cyclin D1-R | CTCTTCTGTCAAGGACTT  | 18     | 55.6    |
| Cyclin D2-F | CCGTTCTTGCTCTTGG    | 17     | 55.1    |
| Cyclin D2-R | AGGCACCTGTTGAAACTGA | 19     | 53.9    |
| Cyclin D3-F | AAACCACGCCCTGACT    | 17     | 57.2    |
| Cyclin D3-R | AGTCCCACCTGAGCTTCC  | 19     | 57.4    |
| Cyclin E-F  | CCCAAGTCTGAGCCAT    | 17     | 54.7    |
| Cyclin E-R  | TCGGAGCCACCTTCTTC   | 17     | 54.5    |

**Dual-Luciferase assay**
Follow our previous method [32]. First, the mouse cyclin D1 promoter sequence was cloned into pGL3-basic plasmid vector to obtain pF1, pF2, pF3, pF4, pF5 and pF6 plasmid. Mutated pF2 was generated by using the Mutant Best Kit (Takara, China). At 48 hours after pAdEasy-Foxg1 or pAdEasy-Myc transfection, the cells were transfected with the above luciferase reporter expression vectors using Lipofectamine 2000 for the promoter assay, respectively. And then, we used a multi-functional microplate reader to detect the expression level of the reporter gene according to the manufacturer's instructions (Promega, E2940).

**EpSCs proliferation assay in vivo**

A deep partial-thickness burn mouse model was made as follows description [34], and EpSCs were labelled with BrdU in mouse skin. Neonatal C57BL/6 mice were intraperitoneally injected with BrdU (50 mg/kg body weight, Sigma) twice daily for 3 days, beginning on day 3 after birth. Skin cells retaining BrdU were identified as EpSCs after 7 weeks. Next, a metal plate (Shandong Academy of Medical Science, China) with a diameter of 1.5 cm and weight of 0.5 kg was used to induce deep partial-thickness burns. The metal plate was heated to 70°C and was placed evenly on the shaved mouse dorsum for 3 sec. Mice were individually housed in plastic cages under standard conditions.

The 36 mice were divided randomly into 6 groups: control (normal saline), pAdEasy-Myc, cyclin D1 siRNA, GDF-5, pAdEasy-Foxg1 + GDF-5, cyclin D1 siRNA + GDF-5. After modeling, control group (normal saline 0.05 ml/g body weight, i.p.), pAdEasy-Myc and pAdEasy-Foxg1 and cyclin D1 siRNA (0.5 ml of PBS containing 2.5×10^8 PFU virus, wound margin five points, s.c.), GDF-5 (0.05 ml/g body weight, i.p.), GDF-5's concentration is 10 µg/ml in normal saline. Mice were sacrificed after 24 hours, and using immunofluorescence assay BrdU and PCNA.

**Statistical methods.**

All data were presented as the mean ± standard deviation (SD) with at least three independent experiments, and analyzed using GraphPad Prism 7.0 software. Statistical significance was evaluated by one-way ANOVA or t-test. \( P<0.05 \) was considered statistically significant.

**Results**

**Effect of GDF-5 on mouse EpSCs proliferation**

Mouse EpSCs were defined as our previously described [31, 35]. The purity of EpSCs was analyzed by flow cytometry, as shown in Fig. 1A, the proportion of EpSCs is about 99%. To illustrate the effect of GDF-5 on mouse EpSCs proliferation, we used CCK-8 assay the proliferation effect of GDF-5 at different time points, and the results showed that 24 h had the best effect on promoting cell proliferation (0.84-fold) when GDF-5 was 100 ng/ml (Fig. 1B), so 24 h was used in the follow-up study. Because the PCNA is a marker that reflects the state of cell proliferation [36], we further analysis of GDF-5 promoting EpSCs proliferation at 24 h found that the proliferation-associated PCNA protein was significant at 100 ng/ml (\( P \))
< 0.01) in response to GDF-5 (Fig. 1C). Based on the above data, GDF-5 concentration of 100 ng/ml and treatment for 24 h were selected for the subsequent experiments.

**The Possible Downstream Molecules Of Gdf-5**

In order to detect the possible downstream molecules of GDF-5, we consulted references and found FOX/cyclin may be the downstream molecules of GDF-5 [19]. Firstly, we screened downstream genes in the FOX family and cyclins, the results discovered that Foxg1 and cyclin D1 were a dose-dependent relationship with GDF-5. In addition, Foxg1 expression increased to 4.79-fold and cyclin D1 increased to 3.31-fold when the concentration of GDF-5 was 100 ng/ml (Fig. 2A). Secondly, the protein levels of Foxg1 and cyclin D1 in mouse EpSCs treated by GDF-5 were detected by WB. The results showed that there was a dose-dependent relationship between cells treated with GDF-5 and Foxg1/cyclin D1 protein expression. Moreover, Foxg1 increased to 1.79-fold and cyclin D1 increased to 1.68-fold when the optimal concentration of GDF-5 was 100 ng/ml (P < 0.01) (Fig. 2B). These results indicated that Foxg1 and cyclin D1 may be the downstream molecules of GDF-5.

**Gdf-5 Promotes Epscs Proliferation Via Foxg1/cyclin D1**

The above part proves that Foxg1 and cyclin D1 are the downstream molecules of GDF-5. Here we discuss the role of Foxg1 and cyclin D1 in GDF-5 promotes cell proliferation. We used adenovirus and siRNA infection technology to verify the interrelationship of Foxg1/cyclin D1 during cell proliferation. The pAdEasy-Myc transfection mouse EpSCs as control virus, the results showed that pAdEasy-Foxg1 and pAdEasy-Myc had a similar transfection efficiency, reaching to 90% and 95% (Fig. 3A), which indicating that each adenovirus successfully infected mouse EpSCs. The pAdEasy-Foxg1 group's Foxg1 expression was reduced by 89% (P < 0.01) compared to the pAdEasy-Myc group, and there was no significance between the pAdEasy-Myc and the control group (Fig. 3B). Three siRNAs were synthesized to inhibit cyclin D1 gene expression. As shown in Fig. 3C, the siRNA1 (P < 0.01) with the best silencing efficiency was selected for subsequent research. Finally, mouse EpSCs were treated with 100 ng/ml GDF-5 or not treated for 24 h, and cell proliferation was evaluated by CCK-8 assay and PCNA protein analysis. Figure 3D and Fig. 3E showed that GDF-5 group had a significant proliferation compared with other groups. In addition, the EpSCs proliferation were inhibited of pAdEasy-Foxg1 + GDF-5 group reduced by 0.78-fold and cyclin D1 siRNA + GDF-5 group reduced by 0.73-fold compared with GDF-5 group (Fig. 3E). These results indicated that pAdEasy-Foxg1 and cyclin D1 siRNA can block the proliferation effect of GDF-5 on EpSCs.

**The effect of GDF-5 on mouse EpSCs proliferation via Foxg1/cyclin D1 in vivo**

To analyze the effect of GDF-5 on EpSCs proliferation in vivo, BrdU-labelled EpSCs and mouse model of burn injury were established as our previously described [29]. BrdU+ and PCNA+ EpSCs were presented by immunochemistry in the regenerated epidermis, and the double positive EpSCs were counted in the
different re-epithelialization area. As can be seen from (Fig. 4A and 4B), the number of double positive cells increased to 40.18-fold in the GDF-5 group compared with the control group, the results showed that GDF-5 can promote EpSCs proliferation in vivo. However, the double positive cells reduced by 16.68-fold and 11.72-fold in the cyclin D1 siRNA + GDF-5 group and the pAdEasy-Foxg1 + GDF-5 group compared with the GDF-5 group, respectively. The data showed that the pAdEasy-Foxg1 and cyclin D1 siRNA abolished the effect of GDF-5 on the number of double positive cells in the regenerated epidermis. Moreover, the double positive cells increased in the pAdEasy-Foxg1 + GDF-5 group and the cyclin D1 + GDF-5 group compared with the control group, but the difference is not obvious. Therefore, we thought that GDF-5 promotes the proliferation of EpSCs through Foxg1 and cyclin D1 in vivo.

**GDF-5 regulates cyclin D1 expression through Foxg1 induces transcriptional activity of the cyclin D1 gene promoter**

In order to analyze the transcription relationship between Foxg1 and cyclin D1, qPCR was used to detect cyclin D1 mRNA and WB was used to detect cyclin D1 protein expression in the presence of pAdEasy-Foxg1. As can be seen from Fig. 5A, the expression of cyclinD1 mRNA in the GDF-5 group increased to 2.34-fold compared with the control group ($P<0.01$), but the cyclin D1 mRNA expression of pAdEasy-Foxg1 + GDF-5 group was reduced back to the control level. At the same time, the cyclin D1 protein expression level also has the same trend (Fig. 5B). This shows that pAdEasy-Foxg1 blocked cyclin D1 expression. Next, we hypothesized that pAdEasy-Foxg1 inhibits cyclin D1 by exerting inhibitory activities to the cyclin D1 promoter. We constructed a pGL3-cyclin D1 (pF1, pF2, pF3, pF4, pF5, pF6) luciferase reporter gene expression vector. The dual luciferase assay revealed that pAdEasy-Foxg1 significantly inhibited the activity of the cyclin D1 promoter (Fig. 5C). However, the mutant pGL3-pF2 did not respond to the pAdEasy-Foxg1 agonists (Fig. 5D). These results suggested that GDF-5-induced cyclin D1 transcription may be regulated by Foxg1-mediated cyclin D1 promoter activity.

**Discussion**

At present, the effect of GDF-5 on wound healing has been reported [13, 15]. However, its specific mechanism for wound repairing is still unclear. In this paper, we discovered that GDF-5 promoted mouse EpSCs proliferation via the Foxg1/cyclin D1 signaling pathway in vivo and in vitro.

Other studies had reported that GDF-5 promote cell proliferation [13], in this study, we found that GDF-5 can directly increase the number of EpSCs in vitro. We detected the effect of GDF-5 on EpSCs when the concentration of exogenous GDF-5 changed from 0 to 1000 ng/ml by CCK-8 assay at 12 hours, 24 hours, 48 hours and 72 hours. The results showed that EpSCs had the best cell proliferation effect after being treated with 100 ng/ml exogenous GDF-5 for 24 hours (Fig. 1B), the effective concentration of GDF-5 on cells is similar to the previously reported [37]. In vivo, through the study of a deep partial-thickness burn mouse model, we found that GDF-5 promoted the proliferation of EpSCs, which is consistent with the results of in vitro experiments (Fig. 4A and 4B). In addition, PCNA is a marker that reflects the state of cell proliferation [36], we tested the expression of PCNA protein after different concentrations of GDF-5
treatment for 24 hours (Fig. 1C). Combining the results of cell count and PCNA protein analysis, it was determined that GDF-5 promoted EpSCs proliferation in vitro and in vivo.

FOX and cyclin have important functions in the proliferation of many cell types [21, 38]. Firstly, we screened several subfamilies of the FOX family and cyclins related to cell proliferation by qPCR. Here, it was found that Foxg1 and cyclin D1 increased significantly (Fig. 2A). Wang fan et al. found that cyclin D1 was significantly expressed during the proliferation of human EpSCs [29]. We previously reported that nitric oxide induces FoxG1 expression in human EpSCs [32]. Analysis on GDF-5 promoting EpSCs proliferation, we found that Foxg1 and cyclin D1 could prevented the proliferation effect of GDF-5 (Fig. 3C, 3D, Fig. 4), further analysis from the protein level found that Foxg1 and cyclin D1 were positively regulated by GDF-5 (Fig. 2B and Fig. 3E). Federica Verginelli et al found that transcriptional program regulated by Foxg1 is significant for promoting glioblastoma growth [39]. Combined with the results of in vivo and in vitro studies, this indicated that after GDF-5 stimulates EpSCs, the downstream molecules Foxg1 and cyclin D1 were activated.

Besides, Foxg1 is involved in inhibiting the cell cycle exit initiated by p21 [27]. Cyclin D1 is a key regulator of cell proliferation by promoting cell cycle transition, and its expression is regulated by transcription level [40, 41]. To clarify the upstream and downstream relationship between Foxg1 and cyclin D1, dual luciferase reporter gene analysis was used, we found that GDF-5 induced cyclin D1 trancription was regulated by Foxg1-mediated cyclin D1 promoter activity (Fig. 5C and 5D). There may be other signaling pathways for GDF-5 to promote EpSCs proliferation. From Fig. 2A discovered Foxo3/Foxp1 decreased significantly and cyclin D2/cyclin D3 increased significantly at the transcription level. Foxo3 and Foxp1 have been reported plays an inhibitory role in cell proliferation [19], cyclin D2/cyclin D3 helps isolate cell transplant factor p27 [42]. Whether GDF-5 regulates these genes to promote the proliferation of EpSCs will be analyzed in another project.

**Conclusions**

This study shows that GDF-5 plays an important role in EpSCs proliferation in vitro and in vivo. The proliferation is regulated by activating Foxg1-cyclin D1 signaling pathway. The results can initially determine that GDF-5 can be used as a new target for wound repair.

**Abbreviations**

EpSCs
Epidermal stem cell
GDF-5
Growth/Differentiation Factor 5
MAPK
Mitogen-activated protein kinase
Wnt
Declarations

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Author Contributions

Xiaohong Zhao and Ruyu Bian performed the experiments and analyzed the data. Fan wang and Ying Wang guided the WB and immunofluorescence assay. Xue Li, Yicheng Guo and Xiaorong Zhang made the necessary corrections in the write up. Gaoxing Luo and Rixing Zhan conceptualized and guided the research project. All authors proof read the manuscript.

Ethics approval and consent to participate

All animal procedures were approved by the Committee on the ethics of Animal Experiments of the Third Military Medical University and were conducted in accordance with the guidelines of the Experimental Animal Department of the Army Military Medical University.

Consent for publication

All patients signed a consent form for their data to be used for research or publication.

Conflict of interest

The authors declare no competing financial interest.

References

1. Beck B, Blanpain C. Mechanisms regulating epidermal stem cells. EMBO J. 2012;31(9):2067–75.
2. Horst BT, Chouhan G, Moiemen NS, Grover LM. Advances in keratinocyte delivery in burn wound care. Adv Drug Deliv Rev. 2017;123:18–32.
3. Iwata Y, Akamatsu H, Hasebe Y, Hasegawa S, Sugiura K. [Skin-resident stem cells and wound healing]. Nihon Rinsho Men'eki Gakkai kaishi = Japanese journal of clinical immunology. 2017;40(1):1–11.
4. Billingham RE, Reynolds J. Transplantation studies on sheets of pure epidermal epithelium and on epidermal cell suspensions. Br J Plast Surg. 1952;5(1):25–36.
5. Li Y, Zhang J, Yue J, Gou X, Wu X. Epidermal Stem Cells in Skin Wound Healing. Advances in Wound Care. 2017;6(9):297–307.
6. Hu P, Yang Q, Wang Q, Shi C, Wang D, Armato U, Prà ID, Chiarini A. Corrigendum to: 'Mesenchymal stromal cells-exosomes: a promising cell-free therapeutic tool for wound healing and cutaneous regeneration'. Burns trauma. 2020;8:tkaa007.
7. Li Z, Maitz P. Cell therapy for severe burn wound healing. Burns & trauma 2018, 6.
8. Chen RE, Thorner J. Function and regulation in MAPK signaling pathways: Lessons learned from the yeast Saccharomyces cerevisiae. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research.
9. Zhu AJ, Haase I, Watt FM. Signaling via β1 Integrins and Mitogen-Activated Protein Kinase Determines Human Epidermal Stem Cell Fate in vitro. Proc Natl Acad Sci USA. 1999;96(12):6728–33.

10. Veltri A, Lang C, Lien WH. Concise Review: Wnt Signaling Pathways in Skin Development and Epidermal Stem Cells. Stem Cells. 2018;36(1):22–35.

11. Thompson CC, Sisk JM 3. Hairless and Wnt signaling: allies in epithelial stem cell differentiation. Cell Cycle. 2006;5(17):1913. rd BG.

12. Bone Morphogenetic Protein (BMP) signaling in development and human diseases. Genes & Diseases. 2014.

13. Schiefer JL, Held M, Fuchs PC, Demir E, Pi?Ger F, Schaller HE, Rahmanian-Schwarz A. Growth Differentiation Factor 5 Accelerates Wound Closure and Improves Skin Quality During Repair of Full-Thickness Skin Defects. Advances in Skin Wound Care. 2017;30(5):223–9.

14. XX Q, Li TG, Hao J, Hu J, Wang J, Simmons H, Miura S, Mishina Y. BMP4 supports self-renewal of embryonic stem cells by inhibiting mitogen-activated protein kinase pathways. Proc Natl Acad Sci USA. 2004;101(16):6027–32.

15. Zaidi SHE, Huang Q, Momen A, Riazi A, Husain M. Growth Differentiation Factor 5 Regulates Cardiac Repair After Myocardial Infarction. J Am Coll Cardiol. 2010;55(2):135–43.

16. Chen X, Zankl A, Niroomand F, Liu Z, Katus HA, Jahn L, Tiefenbacher C. Upregulation of ID protein by growth and differentiation factor 5 (GDF5) through a smad-dependent and MAPK-independent pathway in HUVSMC. Journal of Molecular Cellular Cardiology. 2006;41(1):0–33.

17. Nakahara T, Tominaga K, Koseki T, Yamamoto M, Yamato K, Fukuda J, Nishihara T. Growth/differentiation factor-5 induces growth arrest and apoptosis in mouse B lineage cells with modulation by Smad. Cell Signal. 2003;15(2):181–7.

18. Zeng Q, Li X, Beck G, Balian G, Shen FH. Growth and differentiation factor-5 (GDF-5) stimulates osteogenic differentiation and increases vascular endothelial growth factor (VEGF) levels in fat-derived stromal cells in vitro. Bone. 2007;40(2):0–381.

19. Charvet C, Alberti I, Luciano F, Jacquel A, Bernard A, Auberge P, Deckert M. Proteolytic regulation of Forkhead transcription factor FOXO3a by caspase-3-like proteases. Oncogene. 2003;22(29):4557–68.

20. Myatt SS, Lam EWF. The emerging roles of forkhead box (Fox) proteins in cancer. Nat Rev Cancer. 2007;7(11):847–59.

21. Katoh M, Katoh M. Human FOX gene family (review). Int J Oncol. 2004;25(5):1495–500.

22. Seoane J, Le H-V, Shen L, Anderson SA, Massagué J. Integration of Smad and Forkhead Pathways in the Control of Neuroepithelial and Glioblastoma Cell Proliferation. Cell. 2004;117(2):211–23.

23. Zheng X, Lin J, Wu H, Mo Z, Lian Y, Wang P, Hu Z, Gao Z, Peng L, Xie C. Forkhead box (FOX) G1 promotes hepatocellular carcinoma epithelial-Mesenchymal transition by activating Wnt signal
through forming T-cell factor-4/Beta-catenin/FOXG1 complex. Journal of experimental clinical cancer research: CR. 2019;38(1):475.

24. Zhang S, Zhang Y, Dong Y, Guo L, Zhang Z, Shao B, Qi J, Zhou H, Zhu W, Yan X, et al. Knockdown of Foxg1 in supporting cells increases the trans-differentiation of supporting cells into hair cells in the neonatal mouse cochlea. Cell Mol Life Sci. 2020;77(7):1401–19.

25. Pestell RG. New Roles of Cyclin D1. Am J Pathol. 2013;183(1):3–9.

26. Witzel I-H, Koh LF, Perkins ND. Regulation of cyclin D1 gene expression. Biochem Soc Trans. 2010;38(1):217.

27. Siegenthaler JA, Tremper-Wells BA, Miller MW. Foxg1 haploinsufficiency reduces the population of cortical intermediate progenitor cells: effect of increased p21 expression. Cereb Cortex. 2008;18(8):1865–75.

28. Ma K, Li H, Zhao, Along, Yang. Leilei, Lei, Sun: Overexpression of cyclin D1 induces the reprogramming of differentiated epidermal cells into stem cell-like cells. Cell Cycle 2016.

29. Wang F, Zhan R, Chen L, Dai X, Cao C. RhoA promotes epidermal stem cell proliferation via PKN1-cyclin D1 signaling. Plos One. 2017;12(2):e0172613.

30. Tudor D, Chaudry F, Harper L, Mackenzie IC. The in vitro behavior and patterns of colony formation of murine epithelial stem cells. Cell Prolif. 2007;40(5):706–20.

31. Yao Z, Li H, He W, Yang S, Zhang X, Zhan R, Xu R, Tan J, Zhou J, Wu J. P311 Accelerates Skin Wound Reepithelialization by Promoting Epidermal Stem Cell Migration Through RhoA and Rac1 Activation. Stem Cells Development. 2017;26(6):451–60.

32. Zhan R, Wang F, Wu, Ying, Qian, Wei, Liu, Menglong: Nitric oxide promotes epidermal stem cell proliferation via FOXG1-c-Myc signalling. Nitric Oxide Biology & Chemistry 2018.

33. Zhao X, Zou X, Li Q, Cai X, Li L, Wang J, Wang Y, Fang C, Xu F, Huang Y. Total flavones of fermentation broth by co-culture of Coprinus comatus and Morchella esculenta induces an anti-inflammatory effect on LPS-stimulated RAW264.7 macrophages cells via the MAPK signaling pathway. Microb Pathog. 2018;125:431–7.

34. Zhan R, He W, Wang F, Yao Z, Tan J, Xu R, Zhou J, Wang Y, Li H, Wu J. Nitric oxide promotes epidermal stem cell migration via cGMP-Rho GTPase signalling. Scientific reports. 2016;6(1):30687.

35. Xu ZD, Li HS, Wang S, He WF, Wu J, Luo GX. [Effects of hypoxia inducible factor-1α on P311 and its influence on the migration of murine epidermal stem cells]. Zhonghua shaoshang za zhi = Zhonghua shaoshang zazhi = Chinese journal of burns. 2017;33(5):287–94.

36. Lee KY, Myung K. PCNA modifications for regulation of post-replication repair pathways. Mol Cells. 2008;26(1):5–11.

37. Coleman CM, Vaughan EE, Browe DC, Mooney E, Howard L, Barry F. Growth differentiation factor-5 enhances in vitro mesenchymal stromal cell chondrogenesis and hypertrophy. Stem Cells Dev. 2013;22(13):1968–76.
38. Vezzali R, Weise SC, Hellbach N, Machado V, Heidrich S, Vogel T. The FOXG1/FOXO/SMAD network balances proliferation and differentiation of cortical progenitors and activates Kcnh3 expression in mature neurons. Oncotarget. 2016;7(25):37436–55.

39. Verginelli F, Perin A, Dali R, Fung KH, Stifani S. Transcription factors FOXG1 and Groucho/TLE promote glioblastoma growth. Nat Commun. 2013;4:2956.

40. Camacho-Arroyo I, Hernandez-Hernandez OT. Regulation of Gene Expression by Progesterone in Cancer Cells: Effects on Cyclin D1, EGFR and VEGF. Mini Reviews in Medicinal Chemistry. 2013;13(5):-.

41. Wee P, Wang Z. Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways. Cancers. 2017;9(5):52.

42. Chiles TC. Regulation and Function of Cyclin D2 in B Lymphocyte Subsets. J Immunol. 2004;173(5):2901–7.

Figures

Figure 1
Effect of GDF-5 on mouse EpSCs proliferation in vitro. (A) Flow cytometry analysis of the purity of passaged EpSCs. (B) Mouse primary EpSCs were treated with 0, 1, 50, 100, 500 and 1,000 ng/ml of GDF-5 for 12, 24, 48, 72 hours. Cell proliferation was measured by CCK8. (C) EpSCs were treated by GDF-5 for 24 h, the PCNA levels were analysed by WB. The data were shown as the means ± SD of three independent experiments. *P<0.05 vs. control (0 ng/ml GDF-5 as control), **P<0.01 vs. control.

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Figure 2

Effects of EpSCs were treated by GDF-5 in vitro. Mouse primary EpSCs were treated with 0, 1, 50, 100, 500 and 1,000 ng/ml of GDF-5 for 24 hours. (A) The FOX family and cyclins genes were analysed by qPCR. (B) The Foxg1 and cyclin D1 levels were analysed by WB. The data are shown as the means ± SD of three independent experiments. *P<0.05 vs. control (0 ng/ml GDF-5 as control), **P<0.01 vs. control.
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GDF-5 promotes EpSCs proliferation via Foxg1/cyclin D1 in vitro. Mouse EpSCs were infected with pAdEasy-Myc control adenovirus or pAdEasy-Foxg1. After the cells were transfected for 48 hours with siRNA control plasmid or cyclin D1 siRNAs, mouse EpSCs were treated with 100 ng/ml GDF-5 for 24 hours. (A) Fluorescence effects of pAdEasy-Myc and pAdEasy-Foxg1 infected mouse EpSCs (100x magnification). (B) Foxg1 protein expression level was analysed by WB. Control: non-infected normal mouse EpSCs. (C) Three siRNAs were synthesized to inhibit cyclin D1 expression and quantification of WB. (D) Cell proliferation was measured by CCK-8. (E) The PCNA levels were analysed by WB. The data are shown as the means ± SD of three independent experiments. **P<0.01 vs. Control, ^^P<0.01 vs. siControl, ##P<0.01 vs. the GDF-5 group.
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GDF-5 promotes EpSCs proliferation via Foxg1/cyclin D1 in vitro. Mouse EpSCs were infected with pAdEasy-Myc control adenovirus or pAdEasy-Foxg1. After the cells were transfected for 48 hours with siRNA control plasmid or cyclin D1 siRNAs, mouse EpSCs were treated with 100 ng/ml GDF-5 for 24 hours. (A) Fluorescence effects of pAdEasy-Myc and pAdEasy-Foxg1 infected mouse EpSCs (100x magnification). (B) Foxg1 protein expression level was analysed by WB. Control: non-infected normal mouse EpSCs. (C) Three siRNAs were synthesized to inhibit cyclin D1 expression and quantification of WB. (D) Cell proliferation was measured by CCK-8. (E) The PCNA levels were analysed by WB. The data are shown as the means ± SD of three independent experiments. **P<0.01 vs. Control, ^^P<0.01 vs. siControl, ##P<0.01 vs. the GDF-5 group.
Figure 4

Immunohistochemical analysis of BrdU+ PCNA+ positive mouse EpSCs. The groups including control, pAdEasy-Myc, cyclin D1 siRNA, GDF-5, pAdEasy-Foxg1 + GDF-5 and cyclin D1 siRNA + GDF-5. (A) After BrdU labelling of mouse EpSCs, immunohistochemical analysis for mouse EpSCs. BrdU+ and PCNA+ cells in the regenerated epidermis are shown at the same magnification. Bar = 50 μm. (B) BrdU+ and PCNA+ cells count was performed using Image Pro Plus in the regenerated epidermis. The data are presented as the means ± SD of three independent experiments; **P<0.01 vs. the control group, ###P<0.01 vs. the GDF-5 group.
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GDF-5 regulates cyclin D1 protein and mRNA expression through Foxg1. Mouse EpSCs were infected with pAdEasy-Myc control adenovirus or pAdEasy-Foxg1 and the cells were treated with 100 ng/ml GDF-5 for 24 hours. (A) cyclin D1 mRNA expression, (B) cyclin D1 protein expression. (C and D) mouse EpSCs were transfected with pAdEasy-Myc control adenovirus or pAdEasy-Foxg1 or the luciferase reporter expression vectors or mutated pGL3-pF2 vector using Lipofectamine 2000. The data are presented as the means ± SD of three independent experiments; **P<0.01 vs. the control, ###P<0.01 vs. the GDF-5 group.
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