Epidermal stem cells participate in the repair of scalds via Nanog and Myc regulation

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Abstract. Epidermal stem cells (EpSCs) with high expression of regulatory factor Nanog can promote wound healing. The aim of the present study was to investigate the effectiveness and mechanism of epidermal stem cells (EpSCs) in healing scalds and the underlying molecular mechanism. Mouse EpSCs were isolated from skin tissues and cultured *in vitro*. First, the proliferative ability of EpSCs was determined via the upregulation and downregulation of Nanog expression levels in EpSCs using the MTS-assay. Second, a wound healing assay of the EpSCs with different Nanog expression levels was performed to investigate cell migratory capacities. Third, the protein expression levels of various proteins in EpSCs with Nanog overexpression or knockdown, were determined. Finally, the transfected EpSCs were applied to the rat scald model to observe their effect on scald healing. Subsequently, wound scores, re-epithelialization and capillary density were determined histologically. The results demonstrated that Nanog overexpression enhanced the proliferative ability of EpSCs via cellular (c)-Myc. Moreover, the LV-Nanog group of EpSCs with increased Nanog expression levels exhibited improved healing abilities in the wound healing test than control group. Using western blotting, it was demonstrated that EpSCs that were transfected with a Nanog-overexpression vector expressed high Nanog protein expression levels, whereas small interfering RNA-Nanog-transfected EpSCs exhibited low Nanog protein expression levels. Furthermore, c-Myc expression was synchronized with Nanog expression. It was also revealed that as the expression levels of c-Myc increased, p53 expression levels also increased. In the rat scald model, Nanog-overexpressing EpSCs enhanced wound closure and re-epithelialization. The EpSCs with Nanog knockdown exhibited the opposite effects. The present study therefore indicated that Nanog may have a positive effect on scald healing in rats, which supports its use in EpSC-based treatments against scalds. Furthermore, it was suggested that c-Myc potentially serves a key role in this process and that this process avoids cancerization by relying on the supervision of p53.

Introduction

Stem cell therapy has been reported to be effective for injury healing. Growth, wound healing and cell replacement are considered to be a function of stem cells. The mesenchymal stem cells (MSCs) are the most well studied (1) and can differentiate into epidermal cells of skin appendages, including the sebaceous and sweat glands (2). The potential of stem cells to heal skin wounds is evident. However, basic stem cell transplantation does not guarantee successful skin wound healing (3). Therefore, it is necessary to investigate different therapeutic approaches using stem cells in skin wound healing.

Epidermal stem cells (EpSCs) are located at the epidermal base layer and their potential for stem cell production for tissue repair is significant (4,5). Clinical approaches in wound treatment closely rely on EpSCs to maintain skin homeostasis and facilitate wound healing (6). Moreover, enrichment using EpSCs within cultured epidermal autografts helps treat severe wounds (7,8). The biological activity of stem cells can also further improve wound healing (9). For example, Nanog controls the fate of pluripotent inner cell masses during embryonic development via maintaining pluripotent epiblasts and preventing differentiation (10). Furthermore,

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Nanog, which regulates induced pluripotent stem cell pluripotency and reprogramming, binds to the OCT4 promoter and enhances embryonic stem cell (ESC) self-renewal via mutation (11). Numerous studies have reported that Nanog is a key transcription factor of stem cells (12,13). Our previous study demonstrated that Nanog is inversely related to the differentiation of EpSCs (14). Furthermore, it was also reported that Nanog and the β-catenin/wnt signaling pathway function in EpSCs self-renewal and differentiation (15). Therefore, the aim of the present study was to upregulate Nanog expression and subsequently assess the proliferation of EpSCs. These cells were then used to explore healing in a scalded rat.

Materials and methods

Experimental animals. In total, 15 specific-pathogen-free male Sprague-Dawley (SD) rats (weight, 140-260 g, 6 weeks old) were purchased from Sanxia University (Hubei, China). The rearing environment was relative humidity of 50-60%, and artificial light and dark for 12 h each. The rats were fed in SPF condition for 7 days. All rats were housed at 22-26°C for 21 days. All animal care and experimental procedures were approved by the Ethics Committee of The Third Affiliated Hospital of Guangzhou Medical University (No. 2020122) (Guangzhou, China). All animal experiments were performed in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 2011) (16). All efforts were made to minimize pain and distress of the experimental animals, and all operations comply with the requirements of Guangzhou Medical University on the welfare of laboratory animals, such as operation under anesthesia, euthanasia.

Isolation of mouse EpSCs. Skin tissue was obtained from the back of neonatal SD rats via plastic surgical procedures, washed in PBS and the connective tissue and subcutaneous fat were removed. The skin sample was sterilized with 70% ethanol, rinsed in PBS and minced into 5-mm wide strips using a sharp scalpel. The strips were treated with 0.25% dispase II (Sigma-Aldrich; Merck KGaA) solution at 4°C overnight. The epidermis was mechanically separated from the dermis and incubated in a solution of 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.) at 37°C for 30 min to dissociate the cells. Enzyme activity was subsequently blocked using DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and the cells were suspended with a pipette. The cell suspension was filtered through a stainless-steel mesh attached to a 60-mm cell culture plate to remove any remaining tissue pieces. Subsequently, the cells were transferred to a 15-ml centrifuge tube and collected using centrifugation for 5 min at 800 g and room temperature (RT). To select stem cells, $1x10^6$ dissociated epidermal cells were plated onto collagen type IV (100 μg/ml; Sigma-Aldrich; Merck KGaA)-coated dishes at room temperature for 10 min. The unattached cells were removed and the rapidly adherent epidermal cells were cultured in keratinocyte serum-free medium supplemented with epidermal growth factor, bovine pituitary extract (all from Gibco; Thermo Fisher Scientific, Inc.) and 0.05 mM $CaCl_2$ (Sigma-Aldrich; Merck KGaA).

These cells were cultured at 37°C with 5% CO_2 in a humidified incubator for two days before replacing the medium. The medium was changed every other day. The EpSC isolation procedure was regarded as a standard protocol, which was first reported by Liu *et al* (17) and Jensen *et al* (18). Rat EpSCs were successfully isolated and identified using CK15, CK19 and β1-integrin with immunofluorescence as previously described (14).

Lentivirus vector construction and EpSCs transduction. The coding sequence of rat Nanog were amplified using PCR from the GV208: Rat Nanog primers (Chengdu Jingming Haorui Biotechnology Co. Ltd.) with AgeI/AgeI overhangs, were used and the fragment was cloned into pTZ58 (Fermentas; Thermo Fisher Scientific, Inc.). The AgeI/AgeI fragment was sub-cloned into pUbi and pEGFP-C1 (Takara Bio USA, Inc.) to generate ubiquitin-Nanog-EGFP encoding plasmids. To produce transduced lentivirus, pBABE-puro plasmids were co-transfected along with helper plasmids into 293T cells (ATCC, CRL-3216) and the medium was harvested at 36 and 72 h. The lentiviral was used 3rd generation system. Quantity of lentiviral plasmid used 5 μg for transfection, and the ratio is 3:2:1 ratio of the lentivirus, packaging and envelope plasmids. The MOI is 10, the interval time is 72 h. The lentivirus vector was construction by OriGene Wuxi Biotechnology Co., Ltd. EpSC transduction was performed by incubating the cells in virus-enriched medium for 12 h, which contained 4 μg/ml polybrene. The transduced EpSCs were divided into a lentivirus-Nanog overexpression (LV-Nanog) group and a control group (LV), which was transduced with the control lentivirus vector (OriGene Wuxi Biotechnology Co., Ltd.) which is empty vector. The Nanog primer sequences are: (accession no. NM001100781) forward, CCGTTGGGCTGACATGAG CGT and reverse, GGCAGGCATCGGCGAGGAAT.

Establishment of EpSCs with stable knockdown of Nanog via transfection of small interfering (si)RNA. The isolated EpSCs were cultured at 37°C in a 12-well plate ($5x10^4$ cells/well) for 24 h prior to transfection and the 12-well plate was coated with collagen type IV (100 μg/ml). Transfections were performed using Lipofectamine® RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions for 30 mins at RT. Following transfection, incubated cells for 1-3 days at 37°C. Then, analyze transfected cells and do the subsequent experiments. The following siRNAs were purchased from Qiagen (QIAGEN Shenzhen Company Limited): AllStars Negative Control siRNA (10 μm, cat. no. 1027281); AllStars Hs Cell Death siRNA (10 μm, cat. no. 1027298); Rn_Nanog_2 FlexiTube siRNA (10 μm, cat. no. NM_001100781; GeneGlobe ID, SI02949135). All the siRNA work concentration were kept as 5-10 nM.

Cell proliferation assay. The EpSCs were seeded into 96-well plates, which were coated with collagen type IV, at a density of $2x10^3$ cells/100 μl media/well. The cells were subsequently divided into the following five groups: i) Cells without any treatment; ii) cells transfected with negative control siRNA cells; iii) cells transfected with Nanog-siRNA; iv) cells transduced with LV; and v) cells

transduced with LV-Nanog. In brief, 20 μl CellTiter 96® AQueous One Solution Reagent was added to each well (100 μl media/20 μl MTS reagent; cat. no. G3582; Promega Corporation). Subsequently, 20 μl of MTS was added to each well after seeding on day 1-5 of incubation and cultured in the incubator at 37˚C. After 1 h of incubation, a microplate reader (Thermo Fisher Scientific, Inc.) was used to assess the absorbance at 490 nm. All experiments were repeated independently three times and measurements from three duplicate wells were used to average each sample.

Wound healing assay. The EpSCs were seeded into six-well plates, which were coated with collagen type IV, at a density of $2x10^5$ cells/1,000 μl media/well. The cells were incubated at 37˚C for 24 h to let them adhere to the plate without serum. Scratch wounds were created in confluent cell monolayers using a sterile 200-μl pipette tip and 80% Confluence on either side of the wound at the start of the assay. After 48 h of incubation suspended cells were removed by washing with PBS. Images of the cells were captured immediately using the EVOS Cell Imaging System f1 (Thermo Fisher Scientific, Inc. cat. no: AMF5000). Digitized images of the wounds were analyzed using ImageJ software 3.0 (National Institutes of Health). Wound closure rates were determined as the difference between the wound width at 0 and 48 h.

Western blotting. The control and treated cells were lysed in ice-cold RIPA lysis buffer (Thermo Fisher Scientific, 89900) with protease inhibitors (Thermo Fisher Scientific, 78440) and the protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratories, Inc.) according to the manufacturer's protocol. A total of 30 μg protein/lane was separated by 4-12% NuPage Bis-Tirs gels (Thermo Fisher Scientific, NP0336) and then transferred to a PVDF membranes (Bio-Rad, 1620264) on ice (300 mA for 2 h). The membrane was subsequently blocked with 5% non-fat milk dissolved in TBST (10% TBS, 1% tween) buffer with for 1 h at room temperature. Subsequently, the membranes were incubated with the following primary antibodies against: Nanog (1:1,000; cat. no. 3580); cellular (c)-Myc (1:1,000; cat. no. 9402); p53 (1:1,000; cat. no. 9282); and β-actin (1:10,000; cat. no. 5125) (all purchased from Cell Signaling Technology, Inc.) overnight at 4˚C. After vigorous washing with TBST (6x5 min), the membranes were subjected to incubation with anti-mouse-IgG-HRP (purchased from Cell Signaling Technology, Inc. 1:1,000; cat. no. 7076) for 1 h at room temperature. The membranes were then washed again with TBST (6x5 min), incubated with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc. cat. no. 32209) and imaged using Bio-Rad equipment. Further analysis, as well as image processing and quantification of the bands, was performed using the program Image Lab3.0 (Bio-rad Laboratories, Inc.).

Establishment of the rat scald model and EpSC treatment. The rats were randomly divided into three groups: the normal EpSCs group, the LV-Nanog EpSCs group, the Nanog-siRNA EpSCs group. The rats were intraperitoneally anesthetized using 3% sodium pentobarbital (60 mg/kg) before scalding. All animal procedures conformed to the Ethics Committee of The
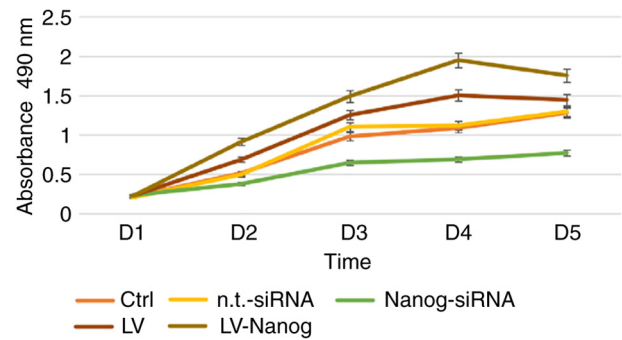

Figure 1. Proliferation effects of Nanog in EpSCs. EpSCs ($5x10^4$) were seeded into 96-wells plates and the absorbance of the cells was detected at day 0-5 in culture medium under the various conditions indicated. Data are presented as the mean ± SEM of four independent wells/condition. EpSC, epidermal stem cell.

Third Affiliated Hospital of Guangzhou Medical University (No. 2020122; Guangzhou, China) A self-made constant temperature and constant pressure scald instrument was used to create the model. Immediately following the scald, 5 ml sodium lactate Ringer's solution was injected intraperitoneally for anti-shock. The burn model was produced by the specialized machine under the following conditions: Temperature, 85˚C; injury pressure of the scald stick, 0.5 kg; scalding head area, 5 cm^2; and scalding time, 8 sec. A stable second-degree scald model was obtained. The area of the scald was 2.25 cm^2 (diameter, 1.5 cm). Each rat had one wound on the middle back and bleeding was stopped by appropriate pressure. On the second day of modeling, the test group was injected with $1x10^5$ EpSCs with different levels of Nanog expression using wet compresses, and the control group was injected with PBS using wet compresses. All wounds received pressure dressings. All the group was injected with 200 μl PBS with cells or without cells.

Sample collection and H&E staining. Images of the scalded areas of five rats in each group were captured on days 0, 5, 10, 14 and 21 following injury. The area of each scald was imaged immediately (data not shown). On day 21 following injury, three rats were randomly selected in each group and anesthetized with 3% sodium pentobarbital. 100 mg/kg sodium pentobarbital was used to sacrifice the animal to make all the rats were dead at the end of the experiment. No rats died unexpectedly during our experiments. Death was verified by the absence of a heartbeat and no breathing. For rodents, overdose of sodium pentobarbital is the most preferable euthanizing agent (19,20). Samples from the backs of the rats were received and fixed at room temperature (RT) using 4% polyphosphate formaldehyde (PFA) for 24 h. The samples were embedded in paraffin and sliced into 3-μm thick sections. Slices were dewaxed with xylene for two times (10 min each), hydrated and stained at RT with hematoxylin for 3-6 min, washed with distilled water for 2 min, treated with a mixture of 1% hydrochloric acid in 70% ethanol for 1-3 sec, stained at RT with eosin for 2-3 min, washed with distilled water for 1-2 sec, hydrated with 80% ethanol and 95% ethanol 15-30 sec each and absolute ethanol 1-2 sec, treated with xylene for 2-3 sec twice. The edges of the tissue slices were cleaned using neutral resin and covered with
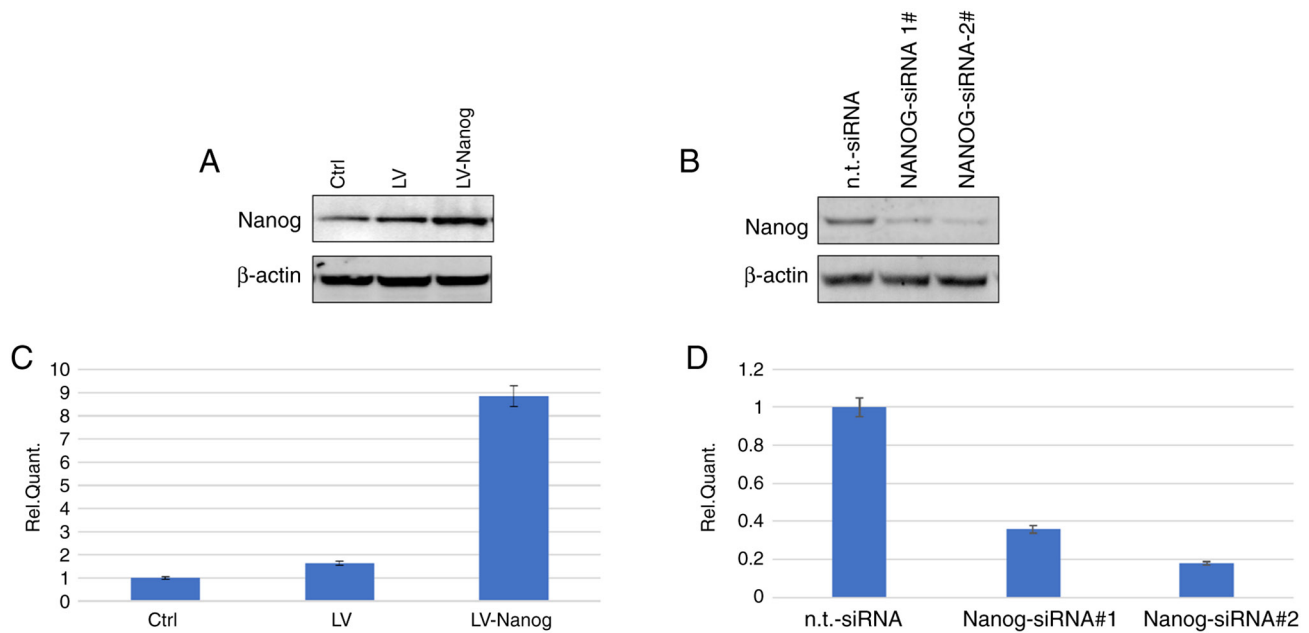
Figure 2. Nanog overexpression and knockdown in EpSCs. (A) Western blotting was used to determine the protein expression levels of the three treatment groups. (B) Protein expression levels of Nanog were downregulated in EpSCs transfected with Nanog-siRNA#1 and #2. Western blotting was used to assess transfection efficiency. (C) Semi-quantification of the western blotting data of LV-Nanog EpSCs. (D) Semi-quantification of the western blotting data of siRNA-Nanog EpSCs. Data are presented as the mean ± SEM (n=3). EpSC, epidermal stem cell; siRNA, small interfering RNA; LV, lentivirus overexpression vector.

coverslips. Pathological changes were observed using a Leica microscope system with light mode and Leica Application Suite X (Leica Microsystems, Inc.).

Statistical analysis. All the experimental repeats three times and the data was analyzed using PRISM 5.0 software (GraphPad Software, Inc.). Data are presented as the mean ± SEM. Since there was no statistical difference in all data analysis between male and female groups, the data from the male and female groups was pooled. The unpaired Student's t-test was used to determine the statistical differences between the control and experimental groups. One-way ANOVA or multifactorial ANOVA were used for comparisons between multiple groups. A Bonferroni post hoc test was used for pairwise comparisons where appropriate. P<0.05 was considered to indicate a statistically significant difference.

Results

EpSCs proliferative ability is influenced by Nanog expression. The proliferative capability of EpSCs was improved when the cells were transduced with LV-Nanog (Fig. 1). Cell proliferation reached its peak on day 4. Compared with the overexpression of Nanog, the cells treated with Nanog-siRNA exhibited significantly reduced proliferation capabilities (P<0.01). Moreover, both the aforementioned increased proliferation and decreased proliferation were significant compared with the vehicle control group (P<0.05). However, the n.t.-siRNA group and LV control group did not significantly affect cell proliferation.

Western blotting analysis of transduced EpSCs. The successful transduction of LV-Nanog EpSCs was confirmed via western blotting following transduction with LV-Nanog for

4 days. The protein expression levels of Nanog were increased in the LV-Nanog EpSCs compared with the LV-control group (Fig. 2A and C).

Western blotting analysis of Nanog-knockdown EpSCs. EpSCs transfected with siRNAs were subsequently analyzed via western blotting. The results demonstrated that the n.t.-siRNA group did not significantly affect the Nanog protein expression levels. However, in the Nanog-siRNA group Nanog protein expression levels were significantly downregulated (Fig. 2B and D).

Nanog expression affects EpSCs wound healing effectiveness. The wound healing assay was used to detect the effect of Nanog on the healing ability of EpSCs *in vitro*. The LV-Nanog EpSCs were demonstrated to heal faster compared with the vehicle control, with the wound being almost completely healed after 48 h (Fig. 3A). Moreover, the EpSCs transfected with Nanog-siRNA were demonstrated to heal slower compared with the vehicle control (Fig. 3B).

Nanog promotes EpSCs proliferation via the activation of c-Myc expression. In the aforementioned results it was demonstrated that LV-Nanog EpSCs exhibited increased proliferation. Subsequently, it was demonstrated via western blotting that Nanog protein expression levels were upregulated and the c-Myc protein expression levels were also upregulated in LV-Nanog group (Fig. 4A and B). In the present study, it was demonstrated that when EpSC proliferation is improved via the upregulation of Nanog expression, c-Myc expression is also upregulated. Furthermore, a similar trend was observed in Nanog-siRNA EpSCs, whereby Nanog is downregulated as well as c-Myc (Fig. 4C). The abnormal upregulation of c-Myc
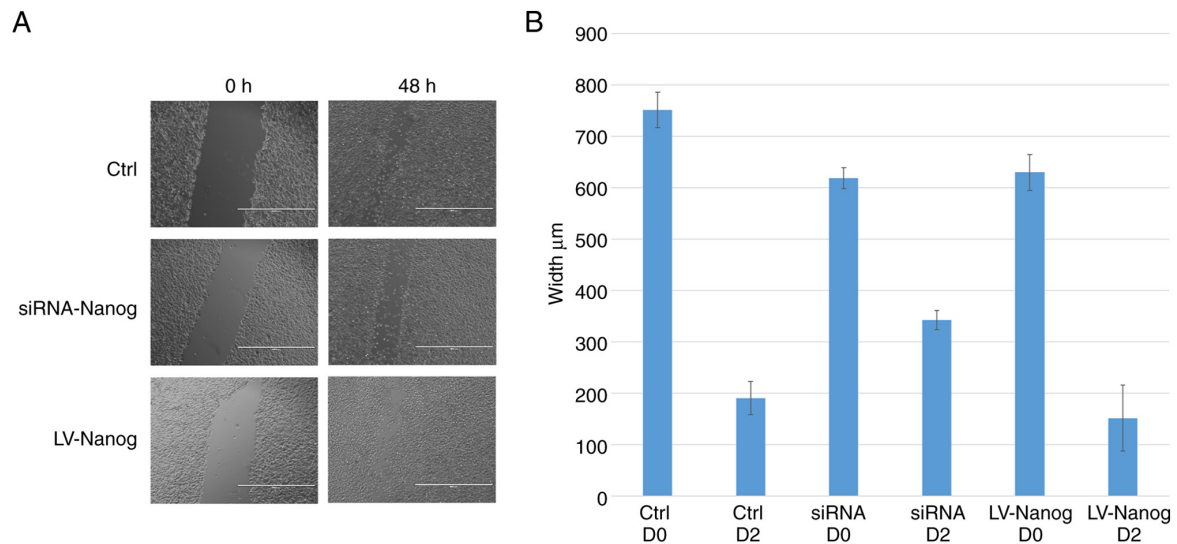
Figure 3. Wound healing assays were used to detect the healing ability of EpSCs with Nanog overexpression or knockdown. (A) The EpSC control group after 48 h exhibited partial wound healing. Wound healing in the EpSCs Nanog-siRNA#1 group could not be observed after 48 h. The wound in the EpSCs LV-Nanog group was almost completely healed after 48 h. (B) Statistical analysis of the width of the wound space in the different groups. was observed using a Leica microscope system with light mode. Scale bar=1000 μm. Data are presented as the mean ± SEM (n=3). EpSC, epidermal stem cell; siRNA, small interfering RNA; LV, lentivirus overexpression vector.
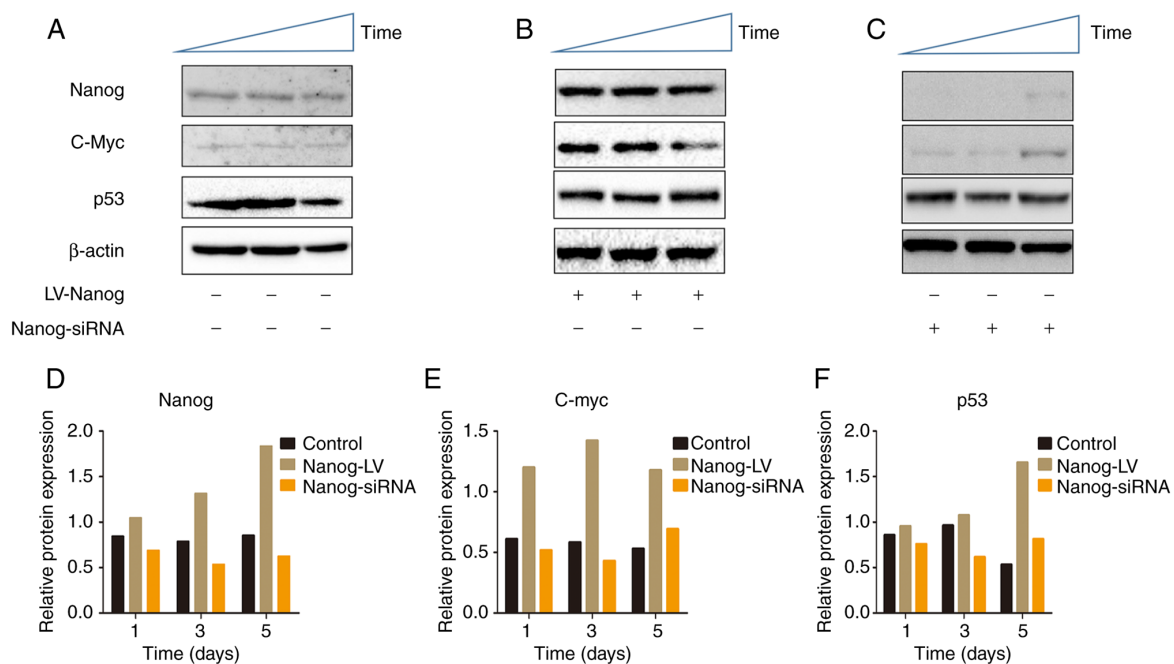

Figure 4. Nanog, c-Myc and p53 proteins were modulated in Nanog overexpressing and knockdown EpSCs. (A) Control EpSCs were seeded into six-well plates coated with collagen type IV (100 μg/ml) at a density of 2.5x10⁵ cells in 100 μl media/well, followed by the addition of epidermal growth factor (0 or 10 ng/ml). The cells were then incubated for 24 h to let the cells adhere to the plate. The lysates harvested were analyzed via western blotting for various proteins using β-actin as a loading control. The Nanog, c-Myc and p53 protein expression levels exhibited no significant difference. (B) In the LV-Nanog EpSCs, Nanog and c-Myc expression levels were upregulated, but both decreased over time. Moreover, p53 expression levels were upregulated over time. (C) In the Nanog-siRNA cell group the Nanog and c-Myc expression levels were downregulated and the p53 was as normal. (D-F) Semi-quantification of the western blotting analysis of the different aforementioned groups. EpSC, epidermal stem cell; siRNA, small interfering RNA; LV, lentivirus overexpression vector.

expression is directly associated with up to 70% of all human cancers (21,22). The present study demonstrated that c-Myc was upregulated alongside Nanog and potentially improved EpSC proliferation.

The p53 or Arf checkpoints are activated by the persistent expression of oncogenic Myc. Loss of checkpoint regulation via mutations in p53 or Arf demonstrates the full tumorigenic potential of Myc (21). The results of the present study demonstrated that in the LV-Nanog EpSCs c-Myc expression levels were upregulated, along with Nanog expression levels, on day 1 and these peak on day 3. Subsequently, c-Myc expression levels decrease; however, p53 expression levels increase. On day 5, the p53 expression levels were even further upregulated and c-Myc expression levels were decreased compared with
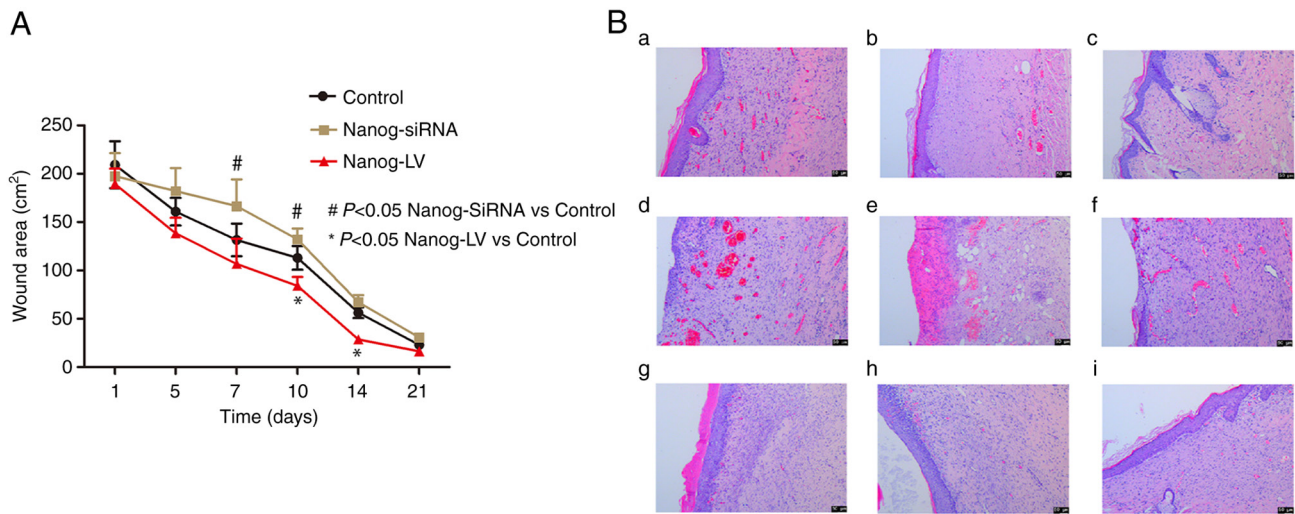
Figure 5. Effect of Nanog overexpression and knockdown in EpSCs on scald healing *in vivo*. (A) The control group of the scald model on days 0, 5, 10, 14 and 21. The Nanog-siRNA group, the LV-Nanog group's scald healing state, Healing curves of different intervention groups. (B) (a-c) Repeated samples within the control group, showed a small number of inflammatory cells and scattered capillaries under the skin via HE staining results of wound tissues on day 21. (d-f) Repeated samples within the Nanog-siRNA group, showed that a large number of inflammatory cells in the subcutaneous tissue, and more scattered capillaries than in the control group via HE staining results of wound tissues on day 21. (g-i) Repeated samples within the Nanog-LV group, showed a dense epidermal layer under the skin, with no obvious capillaries or peripheral inflammatory cells via HE staining results of wound tissues on day 21. H&E staining was observed using a Leica microscope system with light mode. Scale bar=50 μm. Data are presented as the mean ± SEM (n=3). EpSC, epidermal stem cell; siRNA, small interfering RNA; LV, lentivirus overexpression vector.

those on day 3. Moreover, EpSCs proliferation also reached its peak on day 4 and then decreased. These results suggested that p53 expression was affected by c-Myc expression levels, which potentially may help to avoid c-Myc overexpression. Nanog expression levels in the Nanog-siRNA EpSCs were only slightly upregulated on day 5, which may be due to the efficacy of the siRNA decreasing over time. Moreover, the c-myc expression levels were upregulated on D5 slightly. These results supported the hypothesis that an underlying mechanism of Nanog may be to regulate c-Myc expression to improve the proliferation of EpSCs, a process that may be further supervised by p53.

Scald model of rats treated with EpSCs expressing different levels of Nanog. Once the rat scald model was successfully established, wound healing was investigated on days 0, 5, 10, 14 and 21 (data not shown). The LV-Nanog group healed faster, particularly on day 14 (Fig. 5A). Wound healing was observed in H&E-stained scalded tissue specimen on day 21. The wounds in the control group were the most re-epithelialized. Furthermore, the wounds in the LV-Nanog group were almost completely re-epithelialized on day 21, whereas the wounds in the Nanog-siRNA group were only partially re-epithelialized (Fig. 5B).

Furthermore, the epidermal structure was visible in the normal EpSCs group with only a few inflammatory cells visible on day 21. An intact epidermis was visible in the LV-Nanog group and fresh hair follicle tissues were visible in the dermis.

Discussion

It has previously been demonstrated that EpSCs serve a key role in the wound healing process (4,6,23,24). Furthermore, multipotent EpSCs could enrich from keratinocyte isolates,

with their specific location in the hair follicle bulge (5). This result was inconsistent with previous research (15), in this study, the EpSCs proliferation reached its peak on day 4, which was potentially a result of the cell density used and the different of the Nanog expression. Nanog expression maintains EpSC proliferation and the expression of pluripotency genes is maintained by Nanog overexpression (25).

The self-renewal gene, Nanog, is highly expressed in self-renewing embryonic stem cells and has been well studied (12). Nanog is known as one of three 'core' factors that allow for stem cell pluripotency (26). Furthermore. Nanog has been reported to also be expressed in several types of adult stem cells, including EpSCs. However, Nanog is nearly not expressed in differentiated cells. In our previous study, it was demonstrated that Nanog served a key role in the regulation of EpSC proliferation (14,15). Stem cell marker expression, such as that of Nanog and Sox-2, is gradually lost as stem cells age (27). Furthermore, our previous study also reported that Nanog expression is reduced in differentiated cells (14). In the present study, it was demonstrated that high Nanog expression levels improved EpSC proliferation and migration. Previous studies have also suggested that in all Nanog target promoters, genes are equally expressed or repressed, whereas in Nanog unique targets genes were predominantly inactivated or suppressed in response to the expression of a subset of target genes. This observation is common to all factors except Myc (28). In an extended transcriptional network for the pluripotency of ESCs it was demonstrated that Nanog and Myc have a large overlap of target promoters; however, Myc also has its own distinct cluster (28). Myc promotes cell proliferation, as previously demonstrated (29-32). Moreover, dominant negative mutants of Myc antagonizes self-renewal and promotes cell differentiation (29). These results suggested that other transcription factors may be involved in this process.

Myc is a transcription factor that regulates cell proliferation (33). Moreover, Myc is widely expressed during embryogenesis, but also in highly proliferative adult tissues, such as the epidermis and gut (34). Myc is sufficient to stimulate proliferation in quiescent cells via overexpression (35). Furthermore, Myc is known as a co-regulator of cell proliferation and metabolism in numerous types of stem cells (28,36). Therefore, the expression of Myc is finely regulated by transcriptional, post-transcriptional and post-translational regulatory mechanisms in adult tissue homeostasis (37,38). Myc is usually maintained at low levels or restricted to regeneration and cell proliferation, such as in the epidermis and the gut. In mouse ES cells, Myc is required to prevent MAPK activation forcing differentiation of cells into primitive endoderm and serves a role in cell proliferation (30). Together, these aforementioned studies demonstrated that c-Myc functionally upregulates energy production and biosynthetic processes required for successful cellular replication, thereby directly coordinating proliferative metabolism and cell cycle progression. In the present study, the results demonstrated that c-Myc protein expression levels were upregulated when Nanog was overexpressed, which potentially increased EpSC proliferation.

Nanog and Myc, along with Krüppel-like factor 4, are naïve pluripotent markers that drive the transition of EpSCs to ESCs (39). Moreover, Nanog and Myc gene expression are independent of each other in the transcriptional regulatory pathways and do not directly regulate each other, but the Nanog and Myc both stimulate cell proliferation (28). Our previous hypothesis that Nanog overexpression, with high c-Myc expression levels, increased EpSC proliferation, was confirmed by the results of the present study. The result was inconsistent with previous research (12). In this study, the EpSCs reach proliferation peak earlier, which was potentially a result of the cell density used.

Furthermore, in the present study it was demonstrated that Nanog overexpression in EpSCs could potentially improve cell proliferation via enhancing c-Myc expression. However, there is also a protection mechanism to prevent excessive proliferation and tumor development (40).

p53 is a well-known critical tumor suppressor and transcription factor (40,41). In the present study, the results demonstrated that c-Myc protein expression was induced when Nanog was overexpressed in EpSCs, which led to EpSC proliferation. However, overexpression of c-Myc protein can induce hepatocellular carcinoma and other types of tumor (42,43). Co-occupancy regions and cis-overlapping motifs of p53 and c-Myc proteins suggest that these two transcription factors interact and regulate gene expression via competitive binding (44). A previous study also demonstrated that intact p53 suppresses Nanog function (45).

Wound healing is a complex process. Self-repair following an injury occurs underneath the skin and tissue where pluripotent adult stem cells have the ability to self-renew and give rise to different cells types (46). Stem cells give rise to progenitor cells, which cannot self-renew but can give rise to numerous cell types. The extent to which stem cells are involved in skin wound healing is complex and not fully understood (3,46). If epithelium formation in the injured area is rapid, healing will lead to regeneration, or scarring will develop over weeks or months. Therefore, epithelization is the one of the most important factors in wound healing and EpSCs are responsible for the process of epidermalization. The rate of epidermalization also depends on the cell proliferation (47). In the present study, according to the rat scald model, it was demonstrated that following treatment with EpSCs being injected into the wound, the wounds in the LV-Nanog group were re-epithelialized by day 21, which was faster compared with the control group. This led to regeneration that did not result in a scar.

Consequently, to better understand the Nanog-stimulated wound healing, the wound healing assay was used with transfected EpSCs. The results demonstrated that Nanog overexpression in EpSCs increased the wound repair capabilities, whereas in EpSCs with Nanog knockdown wound closure was delayed. Different levels of Nanog in the EpSCs may therefore affect the wound closure rate of the scratch. These results suggested that Nanog potential provides a proliferative advantage to EpSCs and that Nanog may be very active in inducing and supporting the wound healing process.

In the present study, the effectiveness of Nanog on EpSCs during the scald healing process was demonstrated. The data suggested that Nanog overexpression in EpSCs could potentially increase the cell proliferation and enhance the wound closure rate. *In vivo* experiments also supported the hypothesis. When EpSCs expressing LV-Nanog were injected into the rat scald model, the skin re-epithelialized earlier compared with the control group.

In conclusion, in the present study the role of Nanog in EpSC proliferation and migration was investigated. The results demonstrated that in EpSCs, Nanog potentially stimulates cell proliferation and cells that overexpress Nanog may be able to improve wound healing in the rat scald model. Therefore, modifications to EpSC proliferation potential may be an effective way of speeding up the healing of scalds. The present study also demonstrated that the function of Nanog in EpSCs may also be influenced by c-Myc upregulation and p53 control. These data have therefore proposed that this approach could be an effective treatment option to repair wounds to a high quality.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Authors' contributions

DLY performed data curation, software and formal analysis, validation, investigation, provided methodology, acquired

funding, wrote the original draft, wrote reviewed and edited the manuscript. XHZ, QYJ, SL and YL performed data curation, software, formal analysis, validation, investigation and provided methodology. PC conducted software and formal analysis, investigation, wrote, reviewed and edited the manuscript. GQJ conceptualized and supervised the study, provided resources, performed data curation, validation, project administration, wrote, reviewed and edited the manuscript. CYL conceptualized and supervised the study, provided resources, project administration, wrote, reviewed and edited the manuscript. All authors read and approved the final manuscript. DLY and GQJ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

All animal care and experimental procedures in the present study were approved by the Ethics Committee of The Third Affiliated Hospital of Guangzhou Medical University (No. 2020122) (Guangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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