Activation and regulation of the granulation tissue derived cells with stemness-related properties

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Abstract

Introduction: Skin as the largest and easily accessible organ of the body represents an abundant source of adult stem cells. Among them, dermal stem cells hold great promise in tissue repair and the skin granulation tissue has been recently proposed as a promising source of dermal stem cells, but their biological characteristics have not been well investigated.

Methods: The 5-bromo-2′-deoxyuridine (BrdU) lineage tracing approach was employed to chase dermal stem cells in vivo. Granulation tissue derived cells (GTCs) were isolated and their in vitro proliferation, self-renewing, migration, and multi-differentiation capabilities were assessed. Combined radiation and skin wound model was used to investigate the therapeutic effects of GTCs. MicroRNA-21 (miR-21) antagomir was used to antagonize miR-21 expression. Reactive oxygen species (ROS) were scavenged by N-acetyl cysteine (NAC).

Results: The quiescent dermal stem/progenitor cells were activated to proliferate upon injury and enriched in granulation tissues. GTCs exhibited enhanced proliferation, colony formation and multi-differentiation capacities. Topical transplantation of GTCs into the combined radiation and skin wound mice accelerated wound healing and reduced tissue fibrosis. Blockade of the miR-21 expression in GTCs inhibited cell migration and differentiation, but promoted cell proliferation and self-renewing at least partially via a ROS dependent pathway.

Conclusions: The granulation tissue may represent an alternative adult stem cell source in tissue replacement therapy and miR-21 mediated ROS generation negatively regulates the stemness-related properties of granulation tissue derived cells.

Introduction

Stem cell-based therapy has aroused great promise in regenerative medicine and an adult stem cell source is a key resource for clinical application. Skin, the largest organ of the body, is emerging as a reservoir for adult stem cells. Stem cells have been proven to exist in the epithelial layer of the skin including epidermis [1-4] and appendages [5,6]. Recently, dermis – the stromal part of the skin – has been demonstrated as a promising source of stem cell populations with high self-renewing and multilineage differentiation capacities [7]. However, dermis-derived stem cells in normal adults are relatively rare [8]. Very recently, granulation tissue has been proposed as a potential source of dermal stem cells, and stem cells derived from the granulation tissue could improve the recovery of kidney and liver injury [9,10]. However, their biological characteristics were poorly understood. It has been increasingly established that stem cells play an important role in wound healing and granulation tissue formation warrants proliferation and differentiation of dermal stem cells. In this study, we identified that dermal stem/progenitor cells were activated after wounding by the 5-bromo-2′-deoxyuridine (BrdU) lineage tracing approach. Granulation tissue-derived cells (GTCs) were successfully isolated and exhibited enhanced proliferation, colony formation, and multidifferentiation capacities compared with nonwounded adult dermal cells. Topical transplantation of the GTCs accelerated wound healing and reduced tissue fibrosis in mice with combined radiation and skin wound injury. Furthermore, microRNA (miR)-21 and reactive oxygen species (ROS) were significantly upregulated in these cells, and miR-21 was shown to promote cell...
migration and differentiation, but inhibit cell proliferation and self-renewing at least partially via a ROS-dependent pathway.

Methods

Animals

C57/BL mice were obtained from the Center of Experimental Animal at the Third Military Medicine University (TMMU, Chongqing, China). The experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the TMMU, and all procedures were approved by the Animal Care and Use Committee of the TMMU.

Skin wound model

The skin wound model was performed as described previously [11]. In brief, mice were anesthetized with 1% pentobarbital (30 mg/kg), and the back hair was shaved. Circular, full-thickness skin excisions of 10 mm in diameter were surgically made in the middle back of each animal.

Cell isolation and culture

For neonatal and adult dermal cell isolation, dorsal skin was carefully dissected free of other tissue, cut into 1 to 2 mm³ pieces, and washed with phosphate-buffered saline (PBS) three times. After being digested with 0.25% trypsin–ethylenediamine tetraacetic acid (HyClone, Logan, UT, USA) at 4°C overnight, the epidermis was removed, and the remaining dermal parts were further digested with 0.25% collagenase I (Worthington, Biochemical Corporation, Lakewood, NJ, USA), and shaken at 37°C for another 2 hours. The digested cells were then passed through a 75 μm cell strainer (Sangon Biotech, Shanghai, China), centrifuged, and resuspended in Iscove’s Modified Dulbecco’s Media (HyClone), supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/ml penicillin and 0.1 mg/ml streptomycin (all from Beyotime, Shanghai, China). Cells were seeded in a tissue culture flask at 1 × 10³ cells/cm², and maintained at 37°C with 5% carbon dioxide. After 24 hours, the plates were washed with PBS to remove residual nonadherent cells. The remaining adherent cells were subcultured after exposure to 0.25% trypsin–ethylenediamine tetraacetic acid (HyClone) every 3 days. The cells of passage 2 or 3 were used for further experiments. For GTC isolation, the granulation tissues at 7 days after wounding were excised and cut into 1 to 2 mm³ pieces, washed with PBS three times, then digested with 0.25% collagenase I (Worthington, Biochemical Corporation), and shaken at 37°C for 2 hours. Subsequently, they were processed in the same way as described above to harvest the adherent cells. To antagonize the miR-21 expression, cells were pretreated with miR-21 antagonist (50 nM; Ribobio Co., Guangzhou, China) for 48 hours. For scavenging the intracellular ROS, N-acetylcysteine (NAC, 5 mM; Sigma, St. Louis, MO, USA) was added into the culture medium or induced medium.

5-Bromo-2′-deoxyuridine pulse chase

BrdU (100 mg/kg; Sigma), a synthetic nucleoside analog of thymidine, is able to be incorporated into dividing cells. To measure the transient proliferation of the wound tissues, BrdU was injected intraperitoneally 2 hours prior to tissue harvest, and the nonwounded control skin or wounded tissues (n = 3 mice per time point) were excised and fixed in 4% paraformaldehyde at 1, 3, 5, 7, 9, 12, and 15 days after wounding. For label-retaining cell (LRC) detection, BrdU was injected intraperitoneally six times with 12-hour intervals into wounded mice (long-term BrdU labeling group) or into the nonwounded mice (control group). To measure the time of the transient-amplifying cells retaining the BrdU label, BrdU was injected intraperitoneally once at 60 hours after wounding (a single BrdU labeling group). On post-wound days 3, 7, 9, 12, and 15, the wound tissues of the three groups (n = 3 mice per time point for each group) were excised and fixed in 4% paraformaldehyde. The signal of BrdU was detected by immunohistochemistry. For in vitro LRC detection, BrdU was injected intraperitoneally six times with 12-hour intervals immediately after wounding (n = 6 mice) or into the nonwounded mice (n = 6 mice). The nonwounded dermal cells or GTCs at 7 days after wounding were isolated and the adherent cells were reseeded in a six-well plate at a density of 1 × 10³ /well (n = 6 wells per group) and cultured for another 8 days. Subsequently, the cells were fixed in 4% paraformaldehyde, and the colonies were counted. The signal of BrdU was detected by immunofluorescence.

Combined radiation and skin wound model and GTC-based treatment

The total body radiation injury (6 Gy) was made by a 60Co γ-ray source. The absorption rate was 31.02 to 31.98 cGy/minute. Mice were anesthetized with 1% pentobarbital (30 mg/kg) after radiation, and the back hair was shaved. Circular, full-thickness skin excisions of 8 mm in diameter were surgically made in the middle back of each animal. For treatment, 1 × 10⁶ GTCs suspended in 200 μl PBS were intradermally injected around each wound margin. PBS (200 μl) without cells was used as the control. At indicated time points, wounds (n = 6 in the transplantation group or control group) were photographed and quantified by ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA). After the wound healed, the wound repair bed and surrounding tissues were obtained. Paraffin sections were stained by Sirius Red or immunostained with α-smooth muscle actin antibody. The fibrotic tissue depths were quantified in serial sections in the center of wounds treated with GTCs or PBS.
Institutional approval

All procedures on these animals were approved by the Animal Care and Use Committee of the TMMU. All animal experimentation methodology was carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals of the TMMU and the Guidelines on Care and Use of Laboratory Animals issued by the Chinese Council on Animal Research and the Guidelines of Animal Care.

Tissue staining

Immunohistochemistry was performed as described previously [11]. The rabbit anti-BrdU (1:200; Rockland, Philadelphia, Pennsylvania, USA) was used and detected in 3,3’-diaminobenzidine. Slides were counterstained with hematoxylin. The percentage of BrdU-positive cells was calculated by counting the total number of basal cells of granulation tissues and cells expressing nuclear BrdU stain.

Immunofluorescence was also performed as described previously [12]. The rabbit anti-BrdU (1:200; Rockland) or mouse monoclonal anti-a-smooth muscle actin antibody (1:300; Sigma) was used and detected with secondary donkey anti-mouse or rabbit IgG-Cy3 antibody (1:200; Beyotime). Cells were counterstained with the nuclear dye 4’,6-diamidino-2-phenylindole (Beyotime) and examined with a fluorescence microscope.

Sirius red staining was performed as described previously [11]. In brief, paraffin-embedded sections were dehydrated and stained in Sirius red solution for 1 hour, then mounted with Poly-Mount Xylene. Polaroid lens were used for images taken under a microscope.

Quantitative real-time PCR analysis of miR-21 expression

Total RNA was extracted using RNAiso Plus (TaKaRa, Kyoto, Japan). For the miR-21 expression detection, stem-loop RT-PCR was performed as described previously [13]. Quantitative PCR was carried out using a SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) real-time PCR kit (TaKaRa) according to the manufacturer’s protocol. Relative expression was normalized to the expression of U6 small RNA. The primers for miR-21 and U6 were purchased from Ribobio Co. (Guangzhou, China). Three independent experiments were performed. In each experiment, triplicate procedures were performed for each group.

Measuring the endogenous ROS level

The ROS-sensitive dye 2’,7’-dichlorofluorescin diacetate (10 mM; Beyotime) was used to measure the endogenous cellular ROS level according to the manufacturer’s protocol. In brief, the primary cells were incubated with 2’,7’-dichlorofluorescin diacetate for 20 minutes at 37°C with reverse shaking every 3 to 5 minutes. The levels of intracellular ROS were analyzed by measuring the mean fluorescence intensity of 2’,7’-dichlorofluorescin using a flow cytometer as described previously [14]. The data were analyzed by FlowJo 7.6.1 software (Tree-Star, Ashland, OR, USA). Three independent experiments were performed. In each experiment, triplicate procedures were performed for each group.

Cell proliferation assay

Cells were seeded into 96-well plates at 1 × 10^3 cells/well and a volume of 100 µl. A CCK-8 Kit (Dojindo, Kumamoto, Japan) was used to measure the cell proliferation at 0, 2, 4, 6, and 8 days after seeding according to the manufacturer’s recommendations. After incubation for 2 hours, the absorbance was measured at 450 nm by a Model 680 Microplate Reader (Bio-Rad, Hercules, CA, USA). Three independent experiments were performed. Each experiment was performed in sextuplicate for each group.

Colony-forming unit fibroblast assay

Cells (1 × 10^5) were seeded into each well of a six-well plate and incubated for 12 days in a humidified atmosphere (37°C, 5% carbon dioxide). Culture medium was changed every 3 days. Subsequently, cultures were stained with a Giemsa kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s recommendations. Colonies with 50 or more cells in each well of the six-well plate (n = 6 wells for each group) were counted in the microscope. Twenty colonies in each group were randomly selected for mean colony sizes and size distribution analysis. The size of the colonies was measured by Image-J software [15]. Three independent experiments were performed.

Scratch wound closure assay

We used the scratch wound assay to measure the migration of the dermal derived cells as described previously [16]. In brief, 5 × 10^5 cells/well were seeded into six-well plates and cultured for 100% confluence, and then maintained for another day in normal culture medium. A straight line through the cell sheet was made by a p200 pipette tip. Each well was washed with PBS to remove the cells debris and replaced with low serum (2%) culture medium for the cell proliferation inhibition. Two markings created by a razor blade on the outer bottom of each well were used as reference points close to the scratch. Images were captured at the reference points every 12 hours until the wounds were closed (100% confluent) with a microscope (Olympus, Kyoto, Japan). The relative migration distances were measured by Image-J software. Three independent experiments were performed. In each experiment, triplicate was performed for each group.

Differentiation assay

For adipogenic differentiation, cells were seeded into six-well plates with mouse mesenchymal stem cell adipogenic
Figure 1  Activation of dermal stem/progenitor cell proliferation after wounding. (A) At 3, 5, 7, 12, and 15 days after wounding, 5-bromo-2'-deoxyuridine (BrdU) labels in the normal skin tissues or wounded tissues were detected by immunohistochemistry. The percentage of BrdU-positive cells was also calculated, presented as mean ± standard deviation (C). C, control group (n = 3 per time point); d, days after wounding; L, long-term BrdU labeling group (n = 3 per time point); S, a single BrdU labeling group (n = 3 per time point). Scale bar = 100 μm. (B) BrdU (100 mg/kg) was injected intraperitoneally for three consecutive days (once per 12 hours) in nonwounded or wounded mice. Nonwounded dermal cells or granulation tissue-derived cells (GTCs) at 7 days post wounding were isolated and adherent cells were harvested for colony-forming assay. BrdU labels in the colonies were detected by immunofluorescence. Scale bar = 500 μm. (D) Quantification of the colonies. CFU, colony-forming unit. **P < 0.01.
differentiation medium (Cyagen Biosciences, Guangzhou, China) according to the supplier’s instructions. After 3 weeks of differentiation, cells were washed with PBS and then fixed in 10% formalin for 20 minutes and stained with Oil-Red O solution for image acquisition. The intracellular Oil-Red O was extracted with isopropyl alcohol for quantification. The absorbance of the extracted Oil-Red O was measured at 490 nm using a Model 680 Microplate Reader (Bio-Rad). For osteogenic differentiation, cells were seeded into 24-well plates with mouse mesenchymal stem cell osteogenic differentiation medium (Cyagen Biosciences) according to the supplier’s instructions. After 3 weeks of differentiation, cells were washed with PBS and then fixed in 10% formalin for 20 minutes and stained with Alizarin Red solution for image acquisition. An osteogenesis assay kit (Millipore, Billerica, MA, USA) was applied to quantify the Alizarin Red according to supplier’s instructions.

Statistical analysis
Statistical analyses were performed using the SPSS 13.0 package (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation. An independent-samples t test was used to determine the significant differences between two groups. Comparisons of multiple groups were performed with one-way analysis of variance with corrections for multiple comparisons. \( P < 0.05 \) was considered statistically significant.

Results
Activation of dermal stem/progenitor cell proliferation after wounding

It has been long accepted that LRCs represent the stem cell populations in the skin [17], brain [18], bladder [19], and so forth. Thus, we chose the BrdU pulse chase approach to identify the activated dermal stem/progenitor cells in vivo. We treated the wounded mice with BrdU for 3 days after wounding. As shown in Figure 1A,C, BrdU-positive cells decreased sharply during the healing process, but were still found in the lower dermal part of the healed wound bed (about 1.03 ± 0.11%). However, no transient-amplifying cells after a single BrdU injection were detected 12 days after wounding (Figure 1A,C). In the nonwounded dermis, no BrdU-positive LRCs or transient-amplifying cells were found (Figure 1A). In addition, we stained the well-known stem cell markers (Sox2, Oct4, CD133) in the...
granulation tissues at 3, 5, 7, 9, 12, and 15 days after wounding, but no positive stained cells were found (data not shown). Further, we isolated the BrdU-labeled GTCs to test the LRCs in vitro. After 8 days of culture, the BrdU-labeled GTCs could form colonies (20.66 ± 2.13; Figure 1D), and BrdU-positive LRCs were also found in some colonies (Figure 1B), while nonwounded dermal cells failed to form colonies (Figure 1D). These results suggested that resident dermal stem/progenitor cells were quiescent in non-wounded dermis, and were activated after wounding.

**Stemness-related properties of granulation tissue-derived cells**

Before isolating the GTCs, we measured the transient proliferation of the granulation tissues by the 2 hours BrdU pulse approach. The proliferation response of dermal tissues initiated at 3 days after wounding reached a peak at 7 days after wounding, and diminished at 15 days (Additional file 1). Thus, we chose to isolate GTCs at 7 days after wounding. We then tested their stemness-related properties. GTCs exhibited much higher proliferation capacity than nonwounded dermal cells (Figure 2A). GTCs formed 43.33 ± 3.01 colonies after 12 days culture, while nonwounded cells failed to form colonies (Figure 2B). In adipogenic differentiation assay, GTCs produced more adipogenic phenotypic cells stained by Oil-Red O, while nonwounded dermal cells exhibited fewer phenotypic changes. Further, the optical density level of Oil-Red O in induced GTCs (2.44 ± 0.07) was significantly higher than in nonwounded dermal cells (0.28 ± 0.02; Figure 2C). In osteogenic differentiation
assay, GTCs produced more calcium deposition with strong staining of Alizarin Red (136.43 ± 7.82) than non-wounded dermal cells (116.6 ± 3.36; Figure 2D). These results suggested that the proportion of adult dermal stem cells in nonwounded dermis was very low. After wounding, these cells were activated, enriched in granulation tissues, and exhibited enhanced stemness-related properties.

**Therapeutic effects of GTCs on combined radiation and skin wound injury**

It has been well documented that ionizing radiation can delay the wound healing process [20]. In order to test the therapeutic potential of GTCs, a combined radiation and skin wound injury mouse model was further employed in this study. Results showed that GTC transplantation significantly accelerated wound closure (Figure 3A,B). In addition, quantification of fibrotic tissue depth from day 15 (the time of wounds healed) wounds confirmed a significant reduction in fibrotic tissue in GTC-treated wounds (333.34 ± 57.93 μm) compared with that in PBS-treated wounds (443.21 ± 52.49 μm; Figure 3C,D). Moreover, Sirius Red staining for fibrillar collagens revealed less collagen deposition in GTC-treated wounds (Figure 3E). Further results also showed that the myofibroblast marker α-smooth muscle actin-positive cells were lower in the day 15 wounds of GTC-treated wounds (Figure 3F). These data showed that GTC transplantation could promote wound healing and reduce tissue fibrosis in the combined radiation and skin wound injury, suggesting the therapeutic implications of GTCs in tissue replacement therapy.

**miR-21 is a negative regulator of dermal-derived cells for stemness-related properties**

Our recent study has shown that miR-21 was upregulated in granulation tissues after wounding by in situ hybridization [11]. Here, we detected the miR-21 level in GTCs and found it was significantly (29.8-fold change) increased compared with nonwounded dermal cells (Figure 4A). Thus, we further studied the roles of miR-21 in the stemness-related properties of GTCs by antagonizing miR-21 expression with miR-21 antagomir. Surprisingly, the miR-21 antagomir increased the proliferation ability of GTCs (Figure 5A). Further, the colony-forming unit fibroblast assay showed that miR-21 antagomir-treated GTCs formed more colonies (27 ± 1.26) compared with GTCs with control antagomir.
Figure 5 miR-21 negatively regulates stemness-related properties of granulation tissue-derived cells. Passage 2 granulation tissue-derived cells (GTCs) were pretreated with microRNA (miR)-21 antagomir (50 nM) or control antagomir (50 nM) for 48 hours. (A) The proliferation ability was detected by CCK-8 every 2 days after seeding. (B) Colony-forming assay of GTCs pretreated with miR-21 antagomir and control antagomir. Colonies presented as mean ± standard deviation (n = 6 wells per group). Twenty colonies were randomly chosen in each group for analyzing (C) average colony sizes and (D) size distribution. (E) Representative migration photographs of GTCs pretreated with miR-21 antagomir and control antagomir at indicated time points. (F) Relative migration rate presented as mean ± standard deviation (n = 6 per time point for each group). (G) Adipogenic and (H) osteogenic differentiation of GTCs pretreated with miR-21 antagomir or control antagomir were measured by staining with Oil-Red O and Alizarin Red and quantifying them respectively. CFU, colony-forming units; d, days; h, hours; OD, optical density. *P < 0.05, **P < 0.01. Scale bar = 500 μm.
Figure 6 (See legend on next page.)
(20 ± 2.61; Figure 5B), and the sizes of the colonies were also larger in miR-21 antagonim-treated GTCs (14.61 ± 2.25) compared with GTCs with control antagonim (11.11 ± 2.75; Figure 5C,D). However, miR-21 antagonim reduced migration of GTCs in a scratch wound closure assay (Figure 5E,F), and the adipogenic and osteogenic differentiation were also decreased in miR-21 antagonim-treated GTCs (Figure 5G,H). These results suggested that blockade of miR-21 could decrease the migration and multidifferentiation of GTCs, but promote their proliferation and self-renewing capabilities.

To further confirm whether miR-21 negatively regulates stemness-related properties of dermal derived cells, we verified its effects on a miR-21 knock-in neonatal dermal cell model. miR-21 knock-in neonatal dermal cells showed decreased proliferation compared with wild-type neonatal dermal cells (Figure 6A). In addition, they formed less colonies (12 ± 2.0) compared with wild-type neonatal dermal cells (16.0 ± 3.97; Figure 6B). Moreover, the sizes of the colonies of miR-21 knock-in neonatal dermal cells were also smaller (Figure 6C,D). Further, miR-21 antagonim could significantly reverse the decrease of the proliferation and colony-forming abilities (Figure 6A,B,C,D). However, miR-21 knock-in neonatal dermal cells showed increased migration (Figure 6E,F) and adipogenic and osteogenic differentiation (Figure 6G,H) compared with wild-type neonatal dermal cells, and miR-21 antagonim could reverse that increase (Figure 6E,F,G,H). These results further confirmed that miR-21 could decrease proliferation and self-renewing capabilities, but could promote migration and multidifferentiation of dermal-derived cells.

miR-21 negatively regulates the stemness-related properties of dermal-derived cells via a ROS-dependent pathway

Previous studies have shown that ROS were upregulated at the wound site [21,22], but their functions in wound healing are not fully identified. We measured the ROS level in GTCs, and found it was significantly increased in GTCs (261.55 ± 19.26) compared with nonwounded dermal cells (100.00 ± 6.88; Figure 4B). To determine the relationship between miR-21 and ROS, we antagonized miR-21 expression in GTCs and found that the ROS level decreased significantly (115.14 ± 7.91; Figure 4B). On the other hand, we scavenged intracellular ROS in GTCs with a free radical scavenger (NAC), and the miR-21 expression did not change significantly compared with control GTCs (<0.5-fold change; Figure 4A). We next verified the effect of miR-21 on ROS expression in the miR-21 knock-in neonatal dermal cell model. As shown in Figure 4D, the ROS level in miR-21 knock-in neonatal dermal cells (122.96 ± 12.9) was increased compared with wild-type cells (100 ± 2.12), and miR-21 antagonim reversed the increase. In addition, NAC also did not affect miR-21 expression in miR-21 knock-in neonatal dermal cells (<0.5-fold change; Figure 4C).

Further, to determine whether miR-21 regulates stemness-related properties of dermal-derived cells through a ROS-dependent pathway, we scavenged endogenous ROS in the two miR-21 up-regulated cell populations – GTCs and miR-21 knock-in neonatal dermal cells with NAC – and tested their stemness-related properties. As shown in Figure 7A, NAC-treated GTCs exhibited increased proliferation ability compared to control GTCs. In addition, the formed colonies (42 ± 2.45; Figure 7B) and colony sizes (10.24 ± 2.89; Figure 7C, D) of NAC-treated GTCs were increased compared with control GTCs (30.83 ± 9.15 and 7.68 ± 2.73, respectively). However, the migration (Figure 7E,F) and adipogenic (Figure 7G) and osteogenic (Figure 7H) differentiation of NAC-treated GTCs were decreased compared with control GTCs. Moreover, NAC-treated miR-21 knock-in neonatal dermal cells also showed increased proliferation (Figure 8A) and formed more (Figure 8B) and larger colonies (Figure 8C,D) compared with control miR-21 knock-in neonatal dermal cells. Consistent with GTCs, the migration (Figure 8E,F) and adipogenic (Figure 8G) and osteogenic (Figure 8H) differentiation of NAC-treated miR-21 knock-in neonatal dermal cells were also decreased compared with control miR-21 knock-in neonatal dermal cells. All the above data suggest that miR-21 could regulate stemness-related properties of dermal derived cells at least partially via a ROS-dependent pathway.

Discussion

An ideal stem cell source should be easily accessible, immunologically compatible, capable to rapidly expand in culture, and amenable to stable differentiation or
miR-21 negatively regulates stemness-related properties of granulation tissue-derived cells via a reactive oxygen species-dependent pathway. N-acetyl cysteine (NAC; 5 mM) was added into culture medium and adipogenic and osteogenic induced medium of granulation tissue-derived cells (GTCs) to scavenge the intracellular reactive oxygen species (ROS). (A) Proliferation of GTCs with and without NAC was measured every 2 days after seeding. Colony formation assay including (B) colony numbers, (C) average colony sizes and (D) colony size distribution of GTCs with and without NAC was present. (E) Representative migration photographs of GTCs with and without NAC at indicated time points. (F) The migration rate of the two groups presented as mean ± standard deviation (n = 6 per time point for each group). (G) The quantification and representative photographs of the Oil-Red O staining of the adipogenic differentiation of GTCs with and without NAC. (H) Quantification and representative photographs of the Alizarin Red staining of the osteogenic differentiation of GTCs and GTCs with NAC. CFU, colony-forming units; d, days; h, hours; OD, optical density. *P <0.05; **P <0.01. Scale bar = 500 μm.
Figure 8 (See legend on next page.)
transdifferentiation. Skin, the largest organ of the body with easy accessibility, is a promising reservoir for adult stem cells. In the epidermis, epidermal stem cells [23], hair follicle stem cells, and melanocyte stem cells [6] are resident. Recently, dermis has been proven to contain stem cell populations [24-26]. However, the stem cell proportion in the adult was relatively rare. Previous studies demonstrated that the dermal stem cells decreased in number and capabilities of self-renewing or proliferation with aging [8,27]. Skin frequently suffers from injury, and the resident stem cells are proposed to be activated for proliferation and differentiation by various factors from the wound microenvironment including cytokines, growth factors, and extracellular matrix components released by platelets, leukocytes, and macrophages to fulfill the defects [28]. In this study, we showed that dermal stem/progenitor cells were quiescent in nonwounded skin and were activated after wounding and enriched in the granulation tissue. The isolated GTCs exhibited enhanced proliferation, self-renewing, and multidifferentiation capabilities compared with nonwounded dermal cells. Very recently, studies have explored the potential of granulation tissue-derived stem cells in the tissue repair of liver and kidney [9,10], which further suggests that granulation tissue is a promising source of adult stem cells.

miRNAs are evaluated to regulate the expression of one-third of genes, and their roles in wound healing have already attracted much attention [29-32]. In a recent study, we performed miRNA microarray profiling to estimate the changes of the miRNAs at the stage of granulation formation during wound healing, and found that the expression of at least 54 miRNAs changed significantly [11]. miR-21 was one of the most significantly upregulated miRNAs after wounding, and the increased expression of miR-21 at the wound sites was proposed to be modulated by inflammatory factors especially transforming growth factor beta [33,34]. Previous studies showed that the upregulated miR-21 affected multiple aspects of the healing process by booting re-epithelizationization [32], promoting migration of fibroblasts to the wound site [35], and enhancing collagen deposition [11,36]. In this study, we continued to explore the roles of miR-21 in GTCs. Data showed that miR-21 was upregulated in GTCs. Blockade of miR-21 promoted proliferation and self-renewing of GTCs, but decreased their migration and differentiation, which presented as a negative modulator of the stemness-related properties. Previous studies demonstrated that miR-21 showed similar effects in stromal cells. It has been identified that miR-21 could decrease the proliferation of adipose tissue-derived mesenchymal stem cells [37], but promote adipogenic differentiation [38]. In condition with hypoxia and serum deprivation, overexpression of miR-21 inhibited apoptosis of mesenchymal stem cells [39]. However, miR-21 showed different effects in epithelial cells. In a liver regeneration model, miR-21 promoted hepatocyte proliferation by facilitating rapid cyclin D1 translation [40]. Another study showed that miR-21 promoted the senescence of endothelial progenitors by suppressing Hmg22 expression [41].

It is well established that ROS play important roles in regulating cellular functions such as cell proliferation, migration, differentiation, apoptosis, and death [42-46]. According to previous studies, the ROS level at the wound site was upregulated at the early stage of wound healing [22]. In this study, the endogenous ROS level in GTCs was also increased. Previously, ROS was shown to upregulate miR-21 expression via different pathways in different cell types [47-53]. Interestingly, our data showed that scavenging endogenous ROS did not decrease miR-21 expression. On the contrary, inhibiting miR-21 expression suppressed the ROS level significantly. In other studies, miR-21 was also shown to regulate the endogenous ROS level. In the process of kidney fibrogenesis, miR-21 upregulates ROS level by repressing the mitochondrial inhibitor of ROS generation Mpv17, and enhances oxidative kidney damage [54]. miR-21 also targets superoxide dismutase 3 and tumor necrosis factor alpha, and promotes tumorigenesis to a larger extent by regulating endogenous ROS level [55]. Furthermore, we explored that scavenging endogenous ROS could mimic the effects of miR-21 antagonist on GTC properties, which suggested that miR-21 regulated their stemness-related properties at least partially via a ROS-dependent pathway.

Conclusions
The cell source is a keystone for stem cell-based therapy. Previous studies have demonstrated that skin dermis is an abundant stem cell source and is easily accessible. However, stem cell numbers and plasticity deplete with
aging. This study demonstrated that dermal stem/progenitor cells were activated upon injury and enriched in granulation tissue with enhanced stemness-related properties and therapeutic potential, and the miR-21/ROS pathway negatively regulate stemness-related properties of GTCs. Our results suggest that skin granulation tissue represents a promising stem cell source for tissue repair and regeneration.

Additional file

Additional file 1: Figure S1. Showing transient proliferation of skin dermis after wounding. BrdU (100 µg/ml) was injected intraperitoneally 2 hours before the nonwounded and wounded tissues sampling. Normal skin tissues or wounded tissues (n = 3 per time point) at 1, 3, 5, 7, 9, 12, 15, and 20 days post wounding were harvested and fixed 48 hours in 4% paraformaldehyde and embedded in paraffin. Sections of 4 mm were immunostained with BrdU antibody and color developed by diaminobenzidine, and counterstained with hematoxylin. d, days after wounding. Scale bar = 100 µm.

Abbreviations

BrdU: 5-bromo-2′-deoxyuridine; GTC: granulation tissue-derived cell; LRC: label-retaining cell; miR: microRNA; NAC: N-acetylcysteine; PBS: phosphate-buffered saline; ROS: reactive oxygen species; TMMU: Third Military Medicine University.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

ZC conceived and designed the experiments, analyzed the data, and wrote the paper. TD, XJ, and LT participated in collection and assembly of data. CS participated in conception and design, data analysis and interpretation, helped to draft the manuscript and gave final approval of the manuscript. All authors read and approved the manuscript.

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