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Acute downregulation of miR-155 at wound sites leads to a reduced fibrosis through attenuating inflammatory response

Long-Long Yang [1], Jia-Qi Liu [1], Xiao-Zhi Bai [1], Lei Fan, Fu Han, Wen-Bin Jia, Lin-Lin Su, Ji-Hong Shi, Chao-Wu Tang, Da-Hai Hu *

Department of Burns and Cutaneous Surgery, Xijing Hospital, The Fourth Military Medical University, 127 Changle West Road, Xi'an, Shaanxi 710032, China

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ABSTRACT

Fibrosis, tightly associated with wound healing, is a significant symptomatic clinical problem. Inflammatory response was reported to be one of the reasons. MiR-155 is relatively related with the development and requirement of inflammatory cells, so we thought reduce the expression of miR-155 in wound sites could improve the quality of healing through reduce inflammatory response. To test this hypothesis, locally antagonizing miR-155 by directly injecting antagomir to wound edge was used to reduce the expression of miR-155. We found wounds treated with miR-155 antagomir had an obvious defect in immune cells requirements, pro-inflammatory factors IL-1β and TNF-α reduced while anti-inflammatory factor IL-10 increased. With treatment of miR-155 antagomir, the expression of α-smooth muscle actin (α-SMA), Col1 and Col3 at wound sites all reduced both from mRNA levels and protein expressions. Wounds injected with antagomir resulted in the structure improvement of collagen, the collagen fibers were more regularly arranged. Meanwhile the rate of healing did not change significantly. These results provide direct evidences that miR-155 play an important role in the pathogenesis of fibrosis and show that miR-155 antagomir has the potential therapy in prevention and reduction of skin fibrosis.

1. Introduction

Fibrosis, the abnormal and excessive deposition of fibrous tissue, occurs in many diseases such as liver cirrhosis, pulmonary fibrosis, scleroderma, and wound healing of the skin after trauma or surgery [1]. Fibrosis in the skin can lead to the formation of abnormal scars which can cause significant problems in tissue growth, function, movement and esthetics, and so it is a significant symptomatic clinical problem.

Damage to any tissue triggers a cascade of events that leads to rapid repair of the wound-if the tissue is skin, then repair involves re-epithelialization, formation of granulation tissue and contraction of underlying wound connective tissues [1]. This concerted effort by the wounded cell layers is accompanied by, and might also be partially regulated by, a robust inflammatory response. Clearly, this inflammatory response is crucial for fighting infection. But, aside from this role, exactly what are the functions of the various leukocyte lineages that are recruited with overlapping time courses to the wound site, and might they do more harm than good [2,3]? Recent knockout and knockdown studies suggest that depletion of one or more of the inflammatory cell lineages can even enhance healing [4,5]. Wound healing in the PU.1-null mouse appears to occur in the absence of fibrosis, which does not trigger an inflammatory response [5,6]. In the liver, a recent study showed depletion of macrophages during the CCL4 induced damage phase blocks fibrosis [7]. An improved understanding of the molecular mechanisms that regulate and coordinate the inflammatory response in wounds has potential for improved therapeutic intervention of fibrosis [8]. Emerging studies suggest that miRNAs play a significant role in the immune responses. MiRNAs are known to influence the fate of immune cells as well as to regulate adaptive immune responses such as antigen presentation and T-cell receptor signaling [9]. MiR-155 has been one of the miRNAs particular associated with inflammatory and immune responses [10]. MiR-155 represents a common target of a broad range of inflammatory mediators [11]. Silencing of miR-155 in LPS-treated mice causes marked derepression of the c/ebp isoforms and downregulation of granulocyte colony-stimulating factor (G-CSF) [12]. MiR-155

Abbreviations: ECM, extracellular matrix; TGF-β1, transforming growth factor-β1; α-SMA, α-smooth muscle actin; G-CSF, granulocyte colony-stimulating factor; MMP, matrix metalloproteinase.

* Corresponding author.
E-mail address: xjburnlab@126.com (D.-H. Hu).

[1] These authors contributed equally to this work.

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has emerged as a central regulator of the immune system [13]. Therefore, we thought that we could improve the quality of wound healing through reduce inflammatory responses by decreasing the level of miR-155 at wound sites.

Here, we show that by direct delivery of miR-155 antagomir to skin wound decreased the expression of miR-155. Subsequently, we saw a reduced inflammatory responses and improved wound healing quality while the healing rates were not significantly changed. Our results show valuable insights into the molecular mechanism underlying fibrosis and suggest that down regulation of miR-155 at wound sites could improve the quality of wound healing.

2. Materials and methods

2.1. Animals and wound model of skin

Male, adult C57BL mice, with weights of 20–22 g, were obtained from the Center of Experimental Animal, the Fourth Military Medicine University (FMMU, Xian, China). 40 mice were randomly divided into control group and miR-155 antagomir group. Mice were anesthetized with 1% pentobarbital (30 mg/kg), and the hair on their back was shaved. Circular, full-thickness skin excisions of 6 mm in diameter in the middle of back were aseptically generated. The experiments were conducted in accord with the Guidelines for the Care and Use of Laboratory Animals of FMMU, and the experimental protocols used in this study were approved by the Animal Care Committee of FMMU.

2.2. Treatment wound healing with miR-155 antagomir

The chemically synthesized antagomir (Ribobio Company, China) was used to disturb miR-155 expression. After skin excision, miR-155 antagomir (16 μg dissolved in 100 μL PBS) was directly injected into the surrounding dermis of the wound at four sites at days 0, 1, 3, 5 and 7 after injury, while the control wounds received equal amount of scrambled antagomir. The process of wound healing was digitally photographed at an indicated time. Wound area measurement was performed by digital planimetry using Image J. The wound residual rate was calculated as the ratio between the residual wound area at a given time point and the original wound area × 100%. A minimum of ten mice were used for each time point examined.

2.3. Real-time PCR

Total RNA of cultured cells were extracted using RNA-isolation kit (Takara, Japan). 500 ng of total RNA was reverse-transcribed using Prime Script™ RT reagent Kit (Takara, Japan). The obtained cDNA was then amplified by the Bio-Rad IQ5 real-time system (Bio-Rad, USA), using SYBR® Premix Ex Taq™ Kit (Takara, Japan) with specific primers. The PCR conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 30 s, 60 °C for 10 s, and elongation at 72 °C for 15 s. The results from three independent vials were used to determine the relative expression levels of the target genes, which were normalized against the expression level of GAPDH.

2.4. Western blotting

Protein concentration was determined by BCA assay (Pierce, USA). Western blotting proceeded as previously described. Briefly, equal amounts of protein in the cell lysates were separated with 10% SDS–PAGE and transferred onto PVDF membranes for 45–80 min at 100 V. The membranes were blocked with 5% non-fat dry milk for 3 h at room temperature, and were then incubated with 1:350 diluted anti-α-SMA (Boster, China), anti-Col1 (Abcam, UK) and anti-Col3 (Abcam, UK) at 4 °C overnight, respectively. The membranes were washed four times with TBST, and incubated with 1:3000 diluted HRP-conjugated corresponding secondary antibodies (Boster, China) at 37 °C for 1 h. After being washed four times with TBST, the immunoreactive traces were detected with ECL Kit (Millipore, USA). The intensity of each protein expression on the membranes was scanned by AlphaImager scanner and analyzed by AlphaEase FC image process software. The membranes were striped and then reprobed with 1:350 diluted anti-actin antibodies (Boster, China) for loading control.

2.5. Statistical analysis

Results are presented as the mean ± standard error of three independent experiments (SEM). Statistical differences between groups were analyzed by Student's *t*-test or the Mann Whitney *U* test as appropriate using a SPSS 13.0 program. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Direct delivery of miR-155 antagomir to skin wound reduced the level of miR-155

Topical gene therapies aiming at wound healing, such as direct injection of plasmid, application of liposome-complex small interfering RNA, and application of miRNA inhibitor, have been proved effective and successful. Because antagomirs are more resistant to degradation [14]. We tried to deliver miRNA antagomir in a practical and simple way by direct injecting miRNA antagomir solution into the dermis around wounds. Real time PCR results showed the relative level of miR-155 was robustly increased in wound sites after injury. Direct delivery of miR-155 antagomir to skin wounds reduced the increased levels, at each time point the level of miR-155 were significantly reduced compared with control groups (Fig. 1A, $p < 0.05$). This result tells us direct delivery of miR-155 antagomir to wound sites could reduce the level of miR-155.

3.2. Reduced influx of inflammatory cells in miR-155 antagomir treated wounds

Several leukocyte lineages infiltrate the wound site with varying time courses during the inflammatory response to tissue damage. The two primary cell lineages are neutrophils and macrophages, and both of these can exert profound effects on various aspects of the repair process. Recent knockout and knockdown studies suggest that depletion of one or more of the inflammatory cell lineages can even enhance healing. After direct delivery of miR-155 antagomir to the skin wound, we found that the recruitment of inflammatory cells (Fig. 1D and E) decreased compared with control group (Fig. 1B and C). We have previously evaluated neutrophil influx in miR-155 antagomir treated wounds and here we confirm with an anti-myeloperoxidase (MPO) antibody that their numbers are significantly reduced on days 1 and 2, at a stage when neutrophil numbers are peaking in control wounds (Fig. 1F–H, $p < 0.05$). There is now clear evidence that the macrophage influx at a wound site may be linked to the rate of re-epithelialization and to the eventual extent of scarring at the wound site, so we have investigated macrophage numbers immunohistochemically by using F4/80 antibody against the marker of macrophage maturation. We found that the number of macrophages at miR-155 antagomir treated wound sites was significantly reduced at days 2 and 7 after the injury compared with control wounds (Fig. 1I–K, $p < 0.05$). These data clearly indicate that acute reduce the level of miR-155 at the time of wounding leads to a dramatic subse-

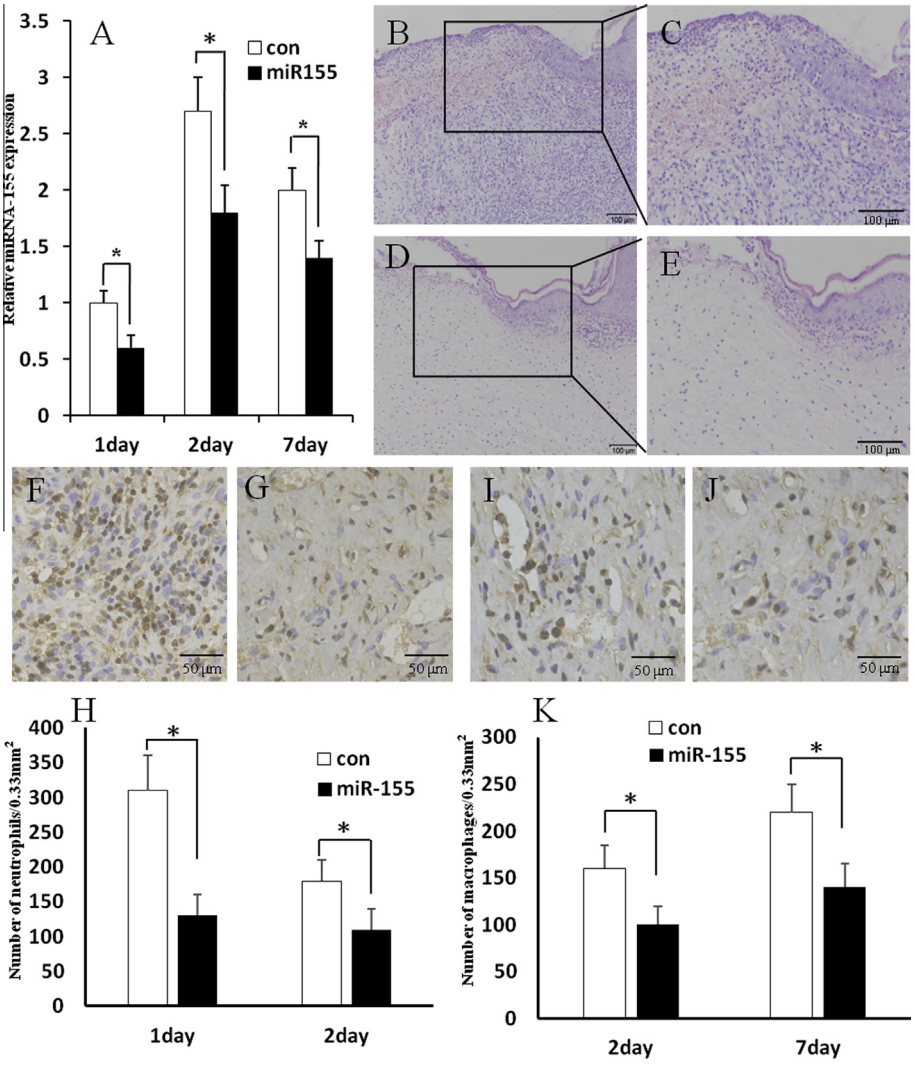

Fig. 1. The expression of miR-155 and inflammatory cells recruitment into wound sites after wounding. (A) The expression of miR-155was robustly upregulated in control wound sites. By comparison, the expression in miR-155 antagomir treated wound sites were significantly reduced ($n = 10$). (B–E) HE staining show the number of inflammatory cells recruitment into the wound sites in miR-155 antagomir treated groups (D and E) were reduced compared with control groups on day 2 (B and C) ($n = 10$). (F and G) Neutrophil recruitment into skin wounds treated with miR-155 antagomir (G) and control group (F) analyzed using an anti-MPO antibody on day 1. (H) Numbers of MPO-positive cells per 0.332 mm^2 decreased significantly in miR-155 treated group compared with control group ($n = 10$) on days 1 and 2. (I and J) Macrophage recruitment into skin wounds treated with miR-155 antagomir (I) and control group (J) analyzed using an anti-F4/80 antibody on day 7. (K) Numbers of F4/80-positive cells per 0.332 mm^2 decreased significantly in miR-155 treated group compared with control group ($n = 10$) on days 2 and 7. Counts are expressed as the mean ± s.e.m.; $^*p < 0.05$.

quent reduction in both the early neutrophil, later macrophage and inflammatory phases.

3.3. Reduced expression of Ccl2, TNF-α, IL-1β and increased expression of IL-10 in miR-155 antagomir treated wounds

Inflammatory cells at the wound site release a large variety of pro-inflammatory cytokines and chemokines that act directly on cells in that site (keratinocytes, fibroblasts and endothelial cells) and amplify the wound inflammatory response, meanwhile they also release a large variety of anti-inflammatory cytokines to reduce the wound inflammatory response. To examine how the reduced influx of inflammatory cells influences the level of these signals, we analyzed Ccl2 as a representative pro-inflammatory chemokine, TNF-α and IL-1β as representative pro-inflammatory cytokines, IL-10 as a representative anti-inflammatory cytokine, respectively. To quantify expression levels of Ccl2, TNF-α, IL-1β and IL-10 we performed real time PCR analysis and ELISA analysis on wound tissue on days 1, 2 and 7. All of the relative mRNA levels were robustly upregulated in control wound sites on day 1, and peaked in expression levels at day 2, after which their levels decreased. By comparison, relative mRNA levels of Ccl2 (Fig. 2A), IL-1β (Fig. 2B) and TNF-α (Fig. 2C) in miR-155 antagomir treated wounds were significantly reduced ($p < 0.05$) on days 2 (Ccl2, TNF-α and IL-1β) and 7 (TNF-α and IL-1β), while relative mRNA level of IL-10 (Fig. 2D) were significantly increased ($p < 0.05$) on day 2. The ELISA results of TNF-α, IL-1β and IL-10 were the same as PCR results, they were robustly upregulated in control group wound sites on day 1, and peaked in levels at day 2, after which their levels decreased. Direct delivery of miR-155 antagomir to skin wound sites significantly reduced ($p < 0.05$) the levels of TNF-α (Fig. 2E) and IL-1β (Fig. 2F) on days 2 (TNF-α, IL-1β) and 7 (IL-1β) and significantly increased ($p < 0.05$) the level of IL-10 (Fig. 2G) on days 2 and 7. These results indicate that reduced recruitment of inflammatory cells in miR-155 antagomir treated wounds was indeed accompanied by changed expression of these signaling molecules without compensation by other cell types.

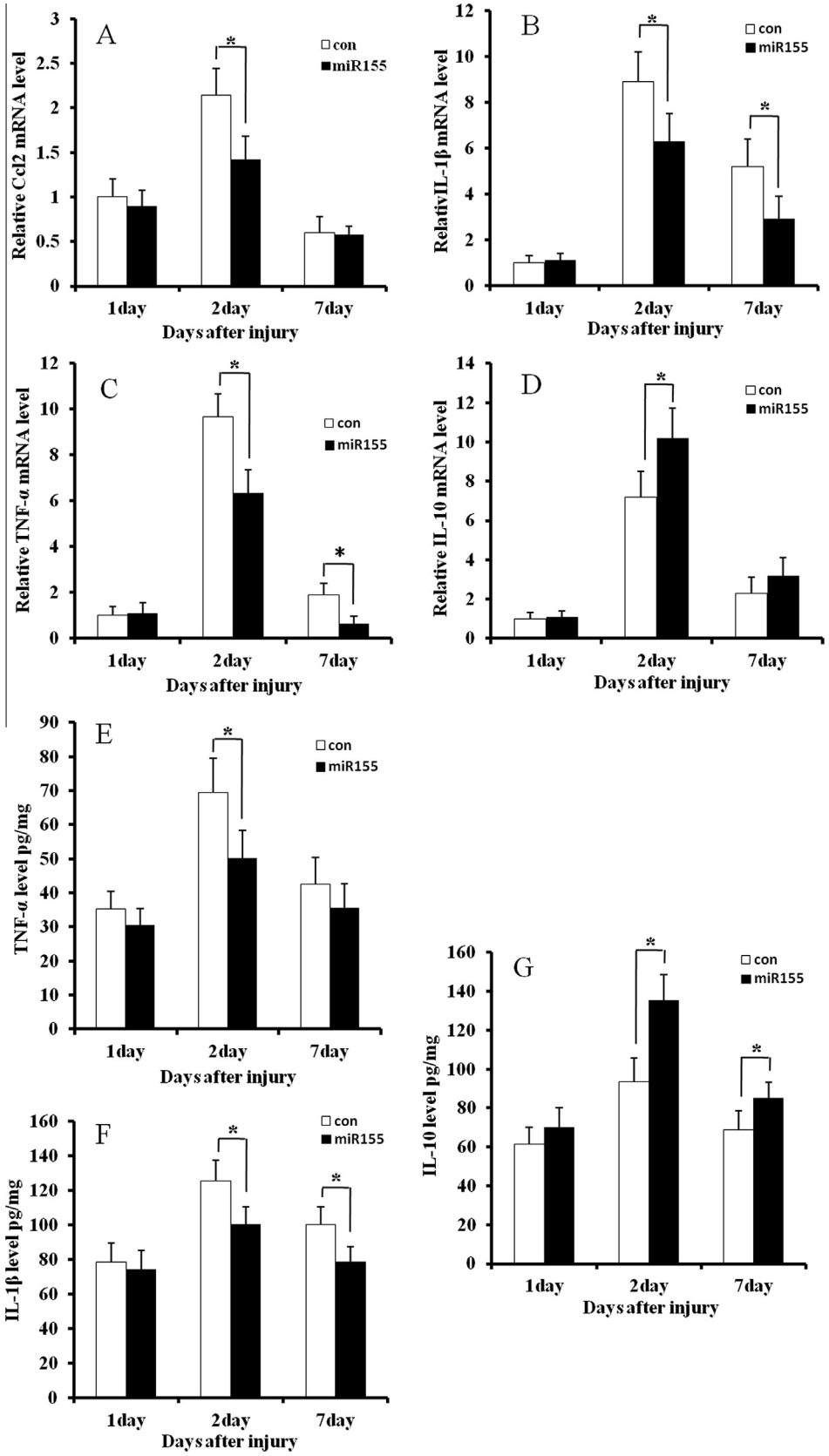

Fig. 2. The expression of Ccl2, TNF-α, IL-1β and IL-10 at wound sites. (A–D) Real-time PCR analysis of the gene expression of Ccl2 (A), IL-1β (B), TNF-α (C) and IL-10 (D) at wound sites. All of the relative mRNA levels were robustly increased in control wound sites on day 1, and peaked in relative levels at day 2, after which their levels decreased. By comparison, relative mRNA levels of Ccl2 (A), IL-1β (B) and TNF-α (C) in miR-155 antagomir treated wounds were significantly reduced on day 2 (Ccl2, TNF-α and IL-1β) and 7 (TNF-α and IL-1β), while relative mRNA level of IL-10 (D) were significantly increased on day 2 ($n = 10$). (E–G) ELISA analysis show us TNF-α (E), IL-1β (F) and IL-10 (G) levels at wound sites were also robustly increased in control group wound sites on day 1, and peaked in levels at day 2, after which their levels decreased. Direct delivery of miR-155 antagomir to skin wound sites significantly reduced the levels of TNF-α (E) and IL-1β (F) on day 2 (TNF-α, IL-1β) and 7 (IL-1β) and significantly increased the level of IL-10 (G) on day 2 and 7 ($n = 10$ for each). Counts are expressed as the mean ± s.e.m.; $^{*}p < 0.05$.

3.4. Direct delivery of miR-155 antagomir to skin wound did not influence the rate of wound healing

To detect whether the decreased recruitment of inflammatory cells and the changed expression of inflammatory cytokines and chemokines caused by direct delivery of miR-155 antagomir to skin wound sites can influence the rate of wound healing, the wound residual rates were calculated as the ratio between the residual wound area at a given time point and the original wound area × 100%. The results show us in control group the wound area decreased with day after wounding and healed at day 9 (Fig. 3). In miR-155 antagomir group, the healing speed was a little slower compared with control group but there was no significantly difference. This showed us direct delivery miR-155 antagomir to wound skin did not influence the rate of wound healing.

3.5. Reduced expression of Col1, Col3 and α-SMA in miR-155 antagomir treated wounds

It is widely accepted that the major characteristics of fibrosis are excessive abnormal deposition and the metabolism disorder of collagen based extracellular matrix (ECM) proteins, mainly including Col1 and Col3, and the transformation of fibroblasts to myofibroblasts (character by α-SMA positive expression). To quantify expression levels of Col1, Col3 and α-SMA we performed real time PCR and Western blotting analysis on wound tissue on day 15 after wounding. Relative mRNA levels of Col1, Col3 and α-SMA (Fig. 4A) were significantly reduced ($p < 0.05$) in miR-155 antagomir treated group compared with control group. Consistent with PCR results, in miR-155 antagomir treated group the protein expressions of Col1, Col3 and α-SMA (Fig. 4B and C) were also significantly reduced ($p < 0.05$). These results tell us direct delivery of miR-155 antagomir to skin wounds improved the quality of wound healing.

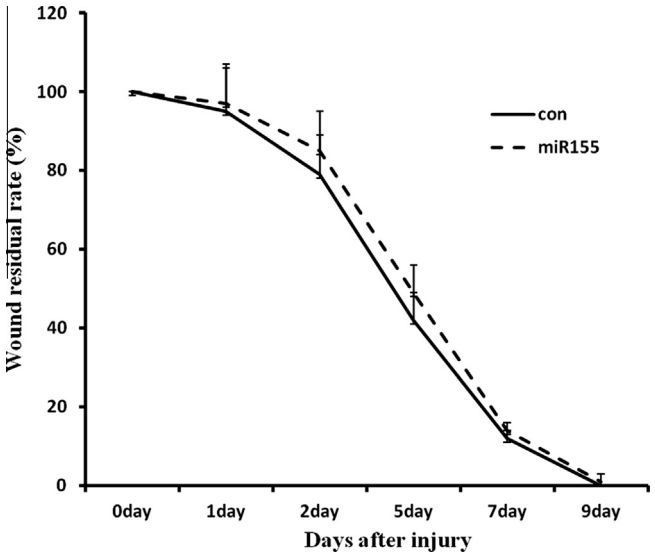

Fig. 3. The rate of wound healing. The process of wound healing was digitally photographed at an indicated time. Wound area measurement was performed by digital planimetry using Image J software version. The wound residual rates were calculated as the ratio between the residual wound area at a given time point and the original wound area × 100%. In control group the wound area decreased with days after wounding and healed at day 9. In miR-155 antagomir group, the healing speed was a little slower compared with control group but there was no significantly difference ($n = 10$). Counts are expressed as the mean ± s.e.m..

3.6. Improved dermal architecture after wound healing in miR-155 antagomir treated wounds

To assess the effects of miR-155 antagomir on wound healing, cutaneous incision wound models were established on BalB/C mice and were treated with subdermal injection of miR-155 antagomir. The wound tissues were harvested 3 weeks later and subject to Masson's staining. As shown, treated with miR-155 antagomir (Fig. 4F and G) resulted in a more disordered structure and denser collagen fibers compared with control (Fig. 4D and E).

4. Discussion

Healing by fibrosis, instead of regeneration, places a huge burden on public health [1]. Importantly, dysfunctional healing often causes lifelong disability, which has a significant economic impact. Thus, if fibrotic healing processes can be transformed into regenerative ones, in which the original tissues are restored, this would considerably improve human health.

Scar formation is the physiological endpoint of mammalian wound repair, which is highly associated with fibrosis. There are different situations which provide evidence that inflammation during the process of wound healing is directly linked to the extent of scar formation. Strikingly, the repair response during the early fetal period is regenerative and scar-less [15,16]. The hallmark of fetal repair is the lack of a typical inflammatory response, suggesting that the absence of inflammation is a prerequisite of regenerative and scar-less repair [16,17]. While there are small numbers of the various leukocyte lineages present in resting tissues, these numbers are massively augmented by recruitment from the circulation in response to inflammatory cues [18,19]. Their prime role appears to be to kill microbes. This is usually achieved in phagolysosomes, but often results in neutrophils blitzing their environs with free radicals that kill many otherwise healthy host cells as well as the target infectious agents [20]. This is particularly apparent in chronic wound situations, and might well underlie the persistent tissue destroying nature of such wounds [7]. In recent years, numerous genetically modified mouse models have advanced our understanding of how the immune response in postnatal life impacts on regeneration and scar formation [21]. Wound healing in the PU.1-null mouse appears to occur in the absence of fibrosis, almost like embryonic wound healing, which is also scar-free and does not trigger an inflammatory response [5,6]. These show us we could improve the wound healing through reduce inflammatory response [3].

Emerging studies suggest that miRNAs play a significant role in the immune responses [9]. MiRNAs are known to influence the fate of immune cells as well as to regulate adaptive immune responses such as antigen presentation and T-cell receptor signaling [22]. MiR-155 has been of particular interest for investigations associated with inflammatory and immune responses [23]. A potential role of miRNA-155 in the adaptive immune response was provided from studies using knockout mice [12]. These miRNA-deficient animals displayed severe immunodeficiencies, particularly impaired B-cell responses and skewed T-cell responses [10]. MiR-155 emerged as a central regulator of lymphocyte differentiation [11]. Thai et al. used a combined genetic loss- and gain-of-function approach, demonstrated that miR-155 regulates specific differentiation processes in the immune response [13]. In our experiments, we used miR-155 antagomir to treat the wound site, we found that inflammatory cells recruitment into the wound sites reduced and inflammatory cytokines also reduced. These data clearly indicate that direct delivery of miR-155 antagomir to wound sites leads to a dramatic subsequent reduced inflammatory response [24].

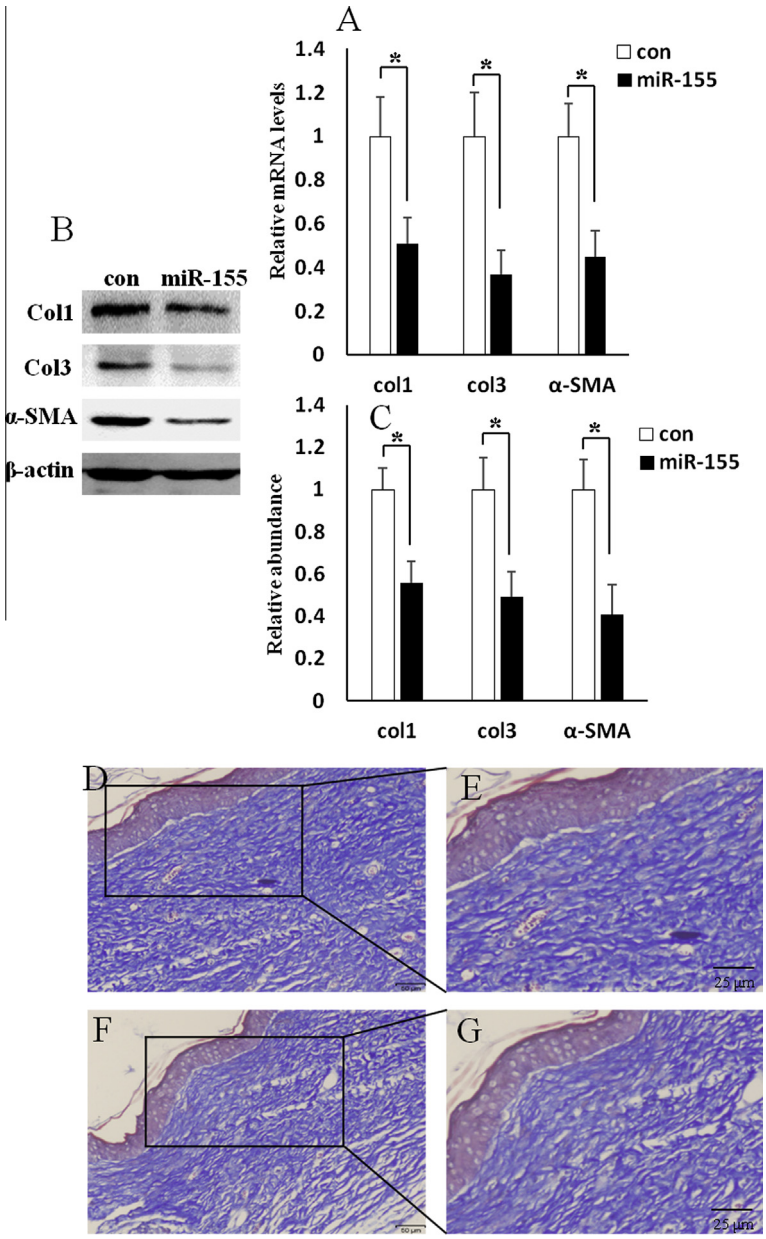

Fig. 4. The expression of Col1, Col3 and α-SMA at wound sites and dermal architecture after wound healing. (A) Relative mRNA levels of Col1, Col3 and α-SMA at wound sites on day 15 after wounding in miR-155 antagomir treatment group were significantly reduced compared with control group ($n = 10$). (B) The protein levels of Col1, Col3 and α-SMA were analyzed by Western blot. (C) Relative quantitative analysis of Col1 protein, Col3 protein and α-SMA protein expressions were significantly reduced in miR-155 antagomir treated group than in control group ($n = 10$). (D–G) The wound tissues were harvested 3 weeks later and subject to Masson's staining. Treatment with miR-155 antagomir (F and G) resulted in a more arranged and thinner collagen fibers compared with control (D and E) ($n = 10$). Counts are expressed as the mean ± s.e.m.; $^{*}p < 0.05$.

It has been well accepted that the major characteristic of hypertrophic scar is metabolic disorder and excessive deposition of collagen-based ECM proteins [25]. In the skin tissue the main types of collagen are Col1 and Col3. Under the normal condition, the dynamic balance between the synthesis and degradation of collagen is regulated by matrix metalloproteinase (MMP), tissue inhibitor of metalloproteinase (TIMP) and various cytokines such as TGF-β1 [17]. The balance is broken after skin injuries, which lead to the increased synthesis and deposition of collagen, then restored to normal when the wound has healed. Dermal fibroblasts are responsible for synthesizing collagen and other ECM proteins, thus they play a critical role in the wound healing and scar formation [12]. After skin injuries, fibroblasts trans-differentiate into myofibroblasts, characterized by expression of α-SMA and the increased ability of collagen synthesis and secretion, and facilitate the wound healing [16]. However, the persistence of myofibroblasts after wound healing may result in the formation of hypertrophic scars. After the reduced inflammatory response in wounds sites treated by miR-155 antagomir we analyzed the expressions of Col1, Col3 and α-SMA to detect whether reduced inflammatory response could improve the quality of wound healing. We found that Col1, Col3 and α-SMA all reduced both from mRNA levels and protein expressions. The regenerated tissues treated with miR-155 antagomir were more regularly arranged in the normal surrounding dermis compared with the control groups. Meanwhile, the rate of wound healing did not changed significantly. These data shown with treatment of miR-155 antagomir the quality of wound healing improved.

Taken together, our present study show that miR-155 antagomir could improve the quality of wound healing by reducing the inflammatory response in wound sites, evidenced by decreased expression of α-SMA, Col1, Col3 and improved the dermal architecture as shown by more arranged and thinner collagen fibers on the mice wound healing model. In addition, the rates of wound healing were not significantly affected. Future studies will focus on the exact molecular mechanisms of the anti-fibrotic properties of miR-155 antagomir.

Conflict of interest

The authors state no conflict of interest.

Acknowledgments

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