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Short Communication

miR-21 Regulates Skin Wound Healing by Targeting Multiple Aspects of the Healing Process

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With the clarification of the important roles of microRNAs (miRNAs) in diverse physiologic and pathologic processes, the effects of miRNAs in wound healing have attracted more attention recently. However, the global pattern of miRNA expression in wound tissue is still unknown. In the present study, we depicted the miRNA profile and identified at least 54 miRNAs, including miR-21, changed for more than twofold at the stage of granulation formation during wound healing. These miRNAs were closely related to the major events of wound healing, including cell migration and proliferation, angiogenesis, and matrix remolding. Furthermore, we found that miR-21 was up-regulated after skin injury, mainly in activated and migrating epithelial cells of epidermis and mesenchymal cells of dermis. Locally antagonizing miR-21 by directly injecting antagomir to wound edge caused significant delay of wound closure with impaired collagen deposition. Unexpectedly, we found wounds treated with miR-21 antagomir had an obvious defect in wound contraction at an early stage of wound healing. The significant role of miR-21 in wound contraction was further confirmed by *in vivo* gain-of-function and *in vitro* loss-of-function experiments. In conclusion, the present study has for the first time depicted miRNA profiling of wound healing and demonstrated the involvement of miR-21 in regulating the wound contraction and collagen deposition. These results suggest that miR-21 may be a new medical target in skin wound manipulation. ***(Am J Pathol 2012, 181:1911–1920; http://dx.doi.org/10.1016/j.ajpath.2012.08.022)***

Wound healing is a complicated process that involves coordinated interactions among an array of tissue repair cells, including fibroblasts, epithelial cells, endothelial cells, and leukocytes. It has been demonstrated that many chemokines, cytokines, and growth factors were secreted mainly by inflammatory cells and the repair cells in wound bed, especially during the time of granulation tissue formation.[1,2] These factors are implicated in the recruitment of inflammatory cells to the wound site and in the activation of tissue repair cells in the vicinity of wound, such as cell proliferation, differentiation, and extracellular matrix synthesis.[1] Although important advances have been made in our understanding of how wound healing progresses in an orderly way, the mechanisms controlling the behavior of repair cells are still not fully understood.

Emerging evidence suggests that microRNAs (miRNAs) play important roles in diverse physiologic and pathologic processes, including development, differentiation, proliferation, apoptosis, and carcinogenesis.[3] However, the roles of miRNAs in wound healing are still unclear because few studies address the relationship between miRNAs and wound healing.[4–9] miR-21 was found to promote keratinocyte migration to accelerate reepithelialization of skin wound.[9] In the present study, using miRNA microarray analysis, we were able to reveal the miRNA signature in wound tissue 7 days after the injury. We found miR-21 expression was up-regulated after wounding, mainly in activated epithelial cells of the epidermis and mesenchymal cells of the dermis. Locally

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antagonizing miR-21 caused significant delay of wound closure accompanied with impaired wound contraction and collagen deposition. These results indicate that wound-induced miR-21 is essential for regulating the skin wound healing process by multiple mechanisms.

Materials and Methods

Animals and Wound Model of Skin

Male, adult C57BL mice, with body weights of 20 to 22 g, were obtained from the Center of Experimental Animal, the Third Military Medicine University (TMMU; Chongqing, China). Mice were anesthetized with 1% pentobarbital (30 mg/kg), and the hair on their back was shaved. Circular, full-thickness skin excisions of 10 mm in diameter in the middle of back or two at each side of the spine were aseptically generated. The experiments were conducted in accord with the Guidelines for the Care and Use of Laboratory Animals of TMMU, and the experimental protocols used in this study were approved by the Animal Care Committee of TMMU.

miRNA Profiling during Wound Healing Revealed by Microarray Analysis

Wound tissue samples, including the wound bed and the margin skin 1 to 2 mm outside the wound, were collected at 4 and 7 days after the creation of skin excision. Normal skin at the corresponding site was sampled as a control. Samples ($n = 3$ each per time point) were embedded in OCT for *in situ* hybridization and in paraffin after 4% paraformaldehyde fixation for H&E staining. Total RNA was extracted from pooled samples ($n = 3$) for quantitative RT-PCR and Northern blot analysis. RNA samples of the normal skin and the wound tissue on day 7 (50 μg each) were sent to CapitalBio Corp. (Beijing, China) for miRNA microarray analysis.

Quantitative Real-Time PCR

Real-time PCR was performed to detect miRNA expression according to the manufacturer protocol of TaqMan microRNA assay kit (Applied Biosystems, Foster City, CA). The PCR results were normalized with U6 small nuclear RNAs as internal control and then expressed as relative expression compared with corresponding control samples. ΔCt was calculated to quantitatively ananlyze the results.

Northern Blot Analysis

The Northern blot analysis was performed as described previously. The relative expression of miR-21 was normalized to U6 small nuclear RNA.

In Situ *Hybridization*

The assay was performed as described previously.[10] Probes for mmu-miR-21 and negative control were digoxigenin-labeled locked nucleic acid–modified detection probes (Exiqon, Copenhagen, Denmark).

Interfering Wound Healing with miR-21 Antagomir

The chemically synthesized antagomir (Ribobio Co., Guangzhou, China) was used to disturb miR-21 expression. After skin excision, miR-21 antagomir (16 μg dissolved in 100 μL of PBS) was directly injected into the surrounding dermis of the wound at three sites. The control wounds received equal amount of scrambled antagomir. The process of wound healing was digitally photographed at an indicated time ($n = 10$). Wound area measurement was performed by digital planimetry using ImageJ software version 2.1.4.6 (NIH, Bethesda, MD). The wound residual rate was calculated as the ratio between the residual wound area at a given time point and the original wound area $\times$ 100%. On days 10 and 16 after wounding, wound tissues were sampled for H&E staining and Masson's staining.

Treatment of Wound with miR-21–Expressing Plasmid

To construct a plasmid expressing miR-21, the genomic fragment containing the miR-21 precursor (~400 bp) was amplified from C57BL mouse genomic DNA and cloned into a pRc-CMV vector (Invitrogen, Carlsbad, CA) downstream CMV promoter. The PCR primers are as follows: 5′-CCCAAGCTTCCCTGTTCATTTTGTTTTGC-3′ and 5′-TGCTCTAGACTTGATACTGCTGCTGTTGT-3′.

After the wound creation of skin excision, 20 μg of miR-21 plasmid DNA (in 200 μL of PBS) was locally injected as above. The empty plasmid was used as control. The wound healing observation and analysis were performed as stated.

Immunohistochemistry for Ki-67

Immunohistochemistry was performed as described previously.[11] The rabbit anti-Ki-67 (1:200; Abcam, Cambridge, UK) was used and detected in 3,3′-diaminobenzidine. To calculate the percentage of Ki-67–positive cells, analyses were performed by counting the total number of basal cells and cells expressing nuclear Ki-67 stain.

Sirius Red Staining and Imaging

Sirius red staining was performed as described previously.[12] In brief, paraffin-embedded slides were dehydrated and stained in Sirius red solution for 1 hour, then mounted with Poly-Mount Xylene. Images were taken under a microscope with and without a Polaroid lens.

Collagen Gel Contraction Assay

The gel contraction assay is based on previously described methods.[13] Briefly, 10T1/2 cells were transfected

with 30 nmol/L miR-21 antagomir or scramble control (Ribobio Co.). After 24 hours in culture, cells were mixed with bovine type I collagen (1.5 mg/mL; Sigma-Aldrich, Dorset, UK) at a concentration of 10^5/mL and aliquot into a 12-well plate. After the cell suspension gelled, an equal volume of Dulbecco's modified Eagle's medium supplemented with 1% bovine serum albumin was added, and the gels were released with a spatula. The gels were maintained for 3 days either in the presence or absence of 2.5 ng/mL of transforming growth factor (TGF)-β1 in culture medium. The capacity of the cells to contract was determined by gel area measurement. Experiments were performed three times in triplicate.

Statistical Analysis

Data were expressed as mean ± SD. The *U*-test was performed to test differences between independent groups at different time points. $P < 0.05$ was considered statistically significant.

Results

miRNA Profile during Granulation Tissue Formation

In the process of wound healing, the granulation tissue formation is a critical stage in which many kinds of growth factors, cytokines, and cells are involved. On our model of skin excisional wound, we chose the day 7 time point to detect the miRNA profile by microarray assay because the granulation tissue contained abundant newly formed capillaries and activated fibroblasts at this stage. Among a total of 743 miRNAs, 33 miRNAs were up-regulated and 21 miRNAs were down-regulated for more than two times of that in normal skin (Figure 1A; see also Supplemental Table S1 at *http://ajp.amjpathol.org*). Some of the miRNAs with changed expression during wound healing were further verified by quantitative PCR. Consistent with the microarray results, the expression of miR-31, miR-21, and miR-203 was up-regulated 17.2, 3.1, and 2.5 times, respectively, whereas miR-249 was down-regulated two times (Figure 1B).

Up-Regulated miR-21 Expression in Wound Tissue during Wound Healing

Because miR-21 was reported to be closely related to the high proliferation ability of many kinds of tumor cells, the increased miR-21 might indicate its involvement in wound healing. We further characterized the increased miR-21 expression in wound tissue by Northern blot. Coincidentally, a high level of miR-21 in wound tissue was confirmed. Compared with the time-matched control, miRNA-21 in wound tissues produced a 6.8- and 8.3-fold increase on the fourth and seventh days after injury (Figure 1, C and D).

In situ hybridization against miR-21 revealed that miR-21 was highly expressed in sebaceous glands and adipose tissue and moderately expressed in cells scattered in the dermis of healthy skin (Figure 1, E–G). However, it was not detectable in epidermis. During wound healing, miR-21 was highly expressed in the epidermis of the skin around the wound, especially at the front of migrating epithelial cells (Figure 1, H and I). In granulation tissue, up-regulated miR-21 expression was also seen extensively in mesenchymal cells (Figure 1J). To our knowledge, this is the first experiment to show the miR-21 location in normal skin and the one during wound healing by *in situ* hybridization. A study close to our project was conducted in our collaborating laboratory, but the study did not report the histologic distribution of miR-21 by *in situ* hybridization.[9]

Direct Delivery of miR-21 Antagomir to Skin Wound Inhibits Wound Healing in Vivo

Although miR-21 expression was significantly increased during cutaneous wound repair, an intervention to reduce miR-21expression is needed to demonstrate the role of miR-21 in skin repair. Topical gene therapies aiming at wound healing, such as direct injection of plasmid,[14] application of liposome-complex small interfering RNA,[15] and application of miRNA inhibitor,[9] have been proved effective and successful. Because antagomirs are more resistant to degradation,[16,17] we tried to deliver miRNA antagomir in a practical and simple way by direct injecting miRNA antagomir solution into the dermis around wounds. After the injection, miR-21 expression level was effectively decreased by 50%, 53%, and 26.7% compared with that with control oligos at 1, 3, and 7 days after injury, respectively (Figure 2A), indicating the injection was practically feasible and effective. Following that, the effect of miR-21 antagomir on the healing process was observed. As early as 1 day after the intervention, the control wounds showed wound contraction. Unexpectedly, the margin skin of miR-21 antagomir–treated wounds became loose and tensionless, which caused approximately one-fourth enlargement of the wound area and a healing delay for 4 to 5 days during the whole process (Figure 2B). It was not until 5 days after the injury that the residual area of miR-21 antagomir–treated wounds was comparable with the initial wound area. There were 80% (8/10) and 100% (10/10) wounds with complete healing in control group at 14 and 18 days, respectively; however, there were only 20% (2/10) and 60% (6/10) in the miR-21 antagomir–treated group, respectively (Figure 2B). This result suggested that miR-21 could be involved in the early contraction of wound and one of the reasons responsible for the delayed healing.

Pathologic Study on Wound Healing

The delay of wound healing in miR-21 antagomir–treated wounds was pathologically confirmed. Comparing the stratification of new epithelium and the cellularity and blood vessel density in granulation tissue, similar pathologic characteristics between miR-21 antagomir–treated wound on day 16 and control wound on day 10 were demonstrated (Figure 2D), which was consistent with the

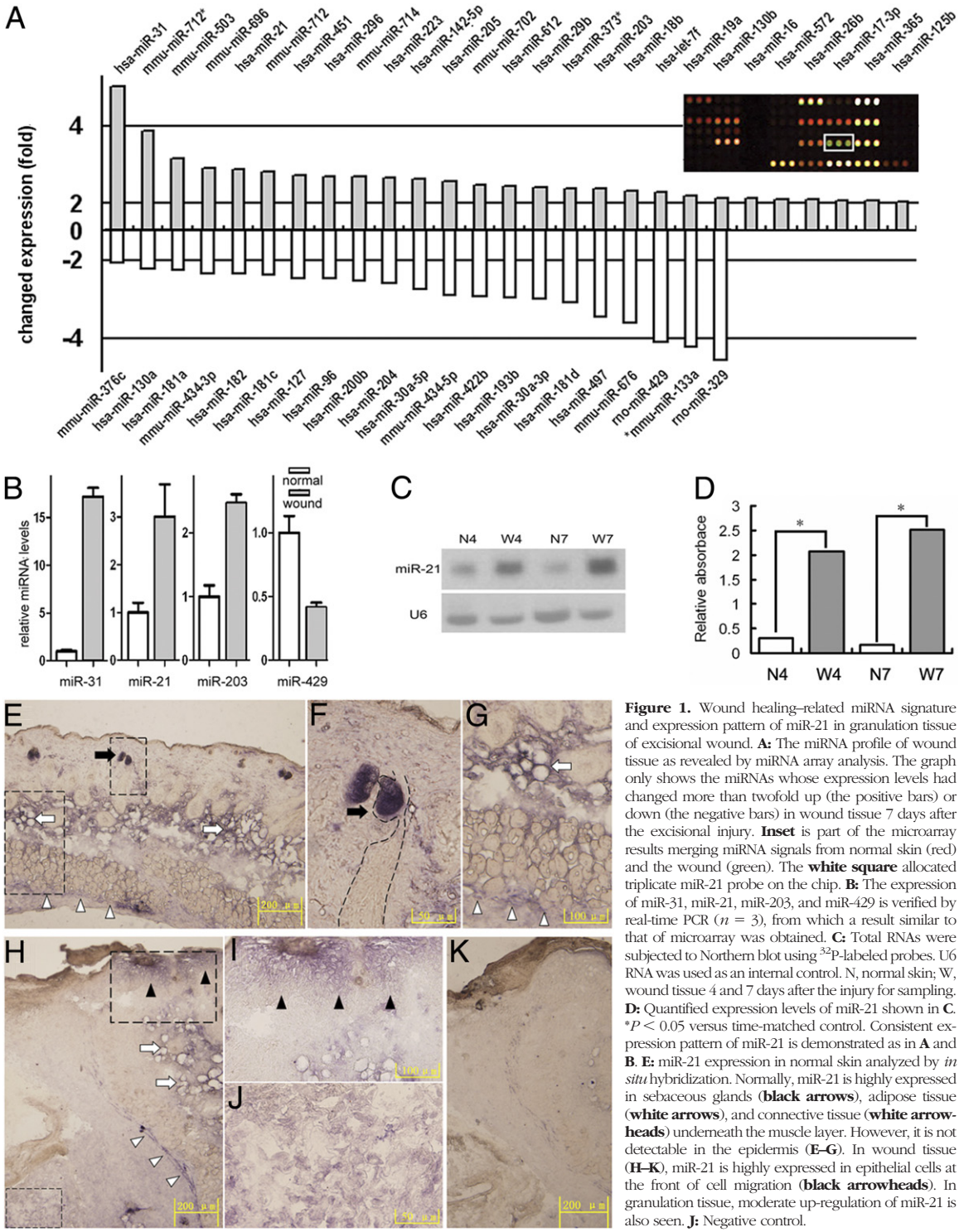

Figure 1. Wound healing–related miRNA signature and expression pattern of miR-21 in granulation tissue of excisional wound. **A:** The miRNA profile of wound tissue as revealed by miRNA array analysis. The graph only shows the miRNAs whose expression levels had changed more than twofold up (the positive bars) or down (the negative bars) in wound tissue 7 days after the excisional injury. **Inset** is part of the microarray results merging miRNA signals from normal skin (red) and the wound (green). The **white square** allocated triplicate miR-21 probe on the chip. **B:** The expression of miR-31, miR-21, miR-203, and miR-429 is verified by real-time PCR ($n = 3$), from which a result similar to that of microarray was obtained. **C:** Total RNAs were subjected to Northern blot using ^{32}P-labeled probes. U6 RNA was used as an internal control. N, normal skin; W, wound tissue 4 and 7 days after the injury for sampling. **D:** Quantified expression levels of miR-21 shown in **C**. $^*P < 0.05$ versus time-matched control. Consistent expression pattern of miR-21 is demonstrated as in **A** and **B**. **E:** miR-21 expression in normal skin analyzed by *in situ* hybridization. Normally, miR-21 is highly expressed in sebaceous glands (**black arrows**), adipose tissue (**white arrows**), and connective tissue (**white arrowheads**) underneath the muscle layer. However, it is not detectable in the epidermis (**E–G**). In wound tissue (**H–K**), miR-21 is highly expressed in epithelial cells at the front of cell migration (**black arrowheads**). In granulation tissue, moderate up-regulation of miR-21 is also seen. **J:** Negative control.

macroview observation. On day 16, the epithelium of control wound exhibited normal thickness of epidermis and recognizable basal layer, granule layer, and horny layer, indicating a satisfactory remodeling. Low cellularity and capillary in the dermis implied well maturation of granulation tissue (Figure 2D). In contrast, the administration of miR-21 antagomir caused a delay of maturation of the epidermis and dermis. The epithelial cells remained in proliferating status, with the characteristics of large cell bodies and nuclei and evident nucleoli. Both the number of cell layers and the thickness of the epidermis were greater than that of the control wound (Figure 2E). The high cellularity (including inflammatory cells and red blood cells) and abundant capillaries suggested the unfinished maturation of granulation tissue (Figure 2D).

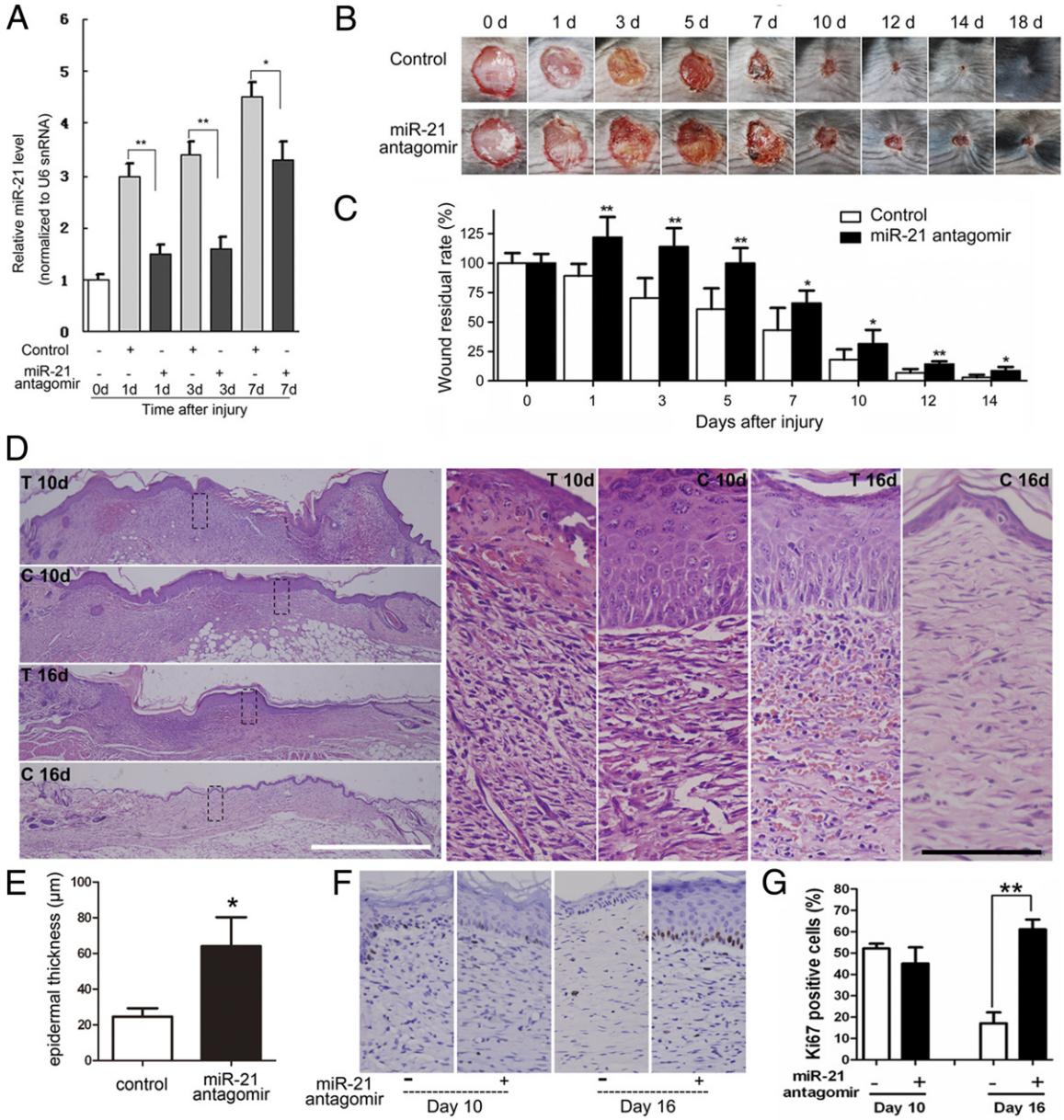

Figure 2. Local treatment with miR-21 antagomir causes significant delay of wound closure. **A:** miR-21 antagomir significantly suppresses the up-regulation of miR-21 in wound tissue ($n = 3$) induced by the injury 1, 3, and 7 day after the injury. **B:** Macroview of the healing of the skin excisional wounds. A representative healing process of miR-21 antagomir treated wound (16 μg in 100 μL of PBS) and its matched control are shown. The wound with anti-miR-21 interference exhibits a wound healing delay. Control, a scramble oligo. **C:** Quantification of wound residual area. The wound residual rate in miR-21 antagomir–treated wounds displays a healing lag for 4 to 5 days from 1 to 14 days. The RNA samples analyzed from pooled wounds ($n = 3$). **D:** Pathologic study confirmed the delayed healing in miR-21 antagomir–treated wounds when comparing the establishment of stratified epidermis, the organization of collagen network, and the development and regression of capillaries during granulation maturation. Dash boxes indicate the areas enlarged correspondingly in the **right panels**. T, miR-21 antagomir treated; C, control; 10 days and 16 days, days after injury. Scale bars: 1 mm (white); 50 μm (black). **E:** The thickness of newly formed epithelium at day 16 ($n = 4$). **F:** The positive cells of Ki-67 staining in newly formed epithelium. At day 10, most of the Ki-67–positive cells are in the basal layer of the epithelium, and some are scattered in the dermis. At day 16, there are few Ki-67–positive cells in normally healed skin; however, many Ki-67–positive cells are in the basal layer of skin from miR-21 antagomir–treated wounds. **G:** The frequency of Ki-67–positive cells in the basal layer of newly formed epithelium ($n = 4$). $^*P < 0.05$, $^{**}P < 0.01$ versus control oligo-treated wound.

Another finding that supported the delayed wound healing in the miR-21 antagomir–treated wound was the ratio of proliferative cells in the basal layer of the new epithelium (Figure 2, F and G). On day 10, in both kinds of wound, most of the Ki-67–positive cells were in basal layer of the epithelium, whereas some were scattered in the dermis. On day 16, there was a significant decline of Ki-67–positive cells in normally healed skin ($P < 0.05$); however, many Ki-67–positive cells were still found in the basal layer of skin from miR-21 antagomir–treated wounds.

miR-21 Antagomir–Inhibited Collagen Deposition and Maturation

Collagen fibril deposition and remodeling are important repair processes in the later phase of granulation tissue

formation. On day 16, the collagen fibers in the dermis of the control wound revealed a typical cross-linked fiber network with vacuous areas (Figures 2D and 3A), which maintains skin tension and elasticity. Correspondingly, the Masson staining pattern in the dermis changed from dense and heavy staining of he 10-day wound to light staining and recognizable collagen network. In the Masson-stained miR-21 antagomir–treated wound on day 16, most of the collagen was homogenously deposited and the fibrils had not organized into the typical network, which was similar to that of control wound on day 10, suggesting a delay of healing process (Figure 3A). When stained with Sirius red, collagen type I produced a red or yellow color, whereas collagen type III turned green under polarized light microscope (Figure 3, B and C). The normal skin near the wound obtained bright Sirius staining in the dermis on days 10 and 16, reflecting that the collagen maturation was a long-lasting process (Figure 3B). On day 10 after injury, there was moderate collagen deposition in the forms of aligned red short fibers. In contrast, it displayed the disordered, shorter, and thinner fibers in green and less red in the miR-21 antagomir–treated wounds. On day 16, the collagen fibers in control granulation tissue matured to form network, but the ones in miR-21 antagomir–treated wounds were still in high density, suggesting a delay in maturation of collagen network (Figure 3C). This finding was consistent with Masson staining of normally healed wound, which exhibited fine collagen network with light color on day 16. Because myofibroblasts have important roles in collagen deposition, we took the 7-day wound sections to analyze the transformation of myofibroblasts. As seen in Supplemental Figure S1 (available at *http://ajp.amjpathol.org*), transient knockdown of miR-21 caused more immature granulation tissue featured by less fibroblasts and more vascular structures. Meanwhile, no significant difference was found between the two kinds of wounds in the staining of α-smooth muscle actin, one essential marker of myofibroblast transformation (see Supplemental Figure S2 at *http://ajp.amjpathol.org*). This result is similar to the skin wound healing processes of *Tgfbr2*^{dermalKO} mice reported previously, which presented less collagen deposition but similar α-smooth muscle actin level compared with the wild-type mice.[13]

miR-21 Is Important for Early Wound Contraction

On examining the wound residual rate, we noticed that wounds treated with miR-21 antagomir had an obvious defect in wound contraction at least at the early stage of wound healing. On day 3, the diameter of the control wound was significantly smaller than that of anti-miR-21–treated wound (Figure 4, A and B). In addition, the migration of the epithelial front was also significantly faster in the control wound, although it contributed less to the healing process at this moment (Figure 4C). To further investigate the role of miR-21 in wound contraction, an *in vivo* gain-of-function experiment was used. After direct injection of miR-21 expressing plasmid to wound edge, the miR-21 expression level was effectively up-regulated by 60% and 62.5% compared with those of empty plasmid-treated control on days 1 and 3, respectively (Figure 4D). Meanwhile, the contraction of the miR-21 overexpressing wounds was significantly stronger than those of control on days 1 and 3 (Figure 4E). H&E-stained 6-day-old wounds also confirmed that transient overexpression of miR-21 enhanced granulation tissue formation as featured by more fibroblast residing (see Supplemental Figure S3 at *http://ajp.amjpathol.org*).

Because TGF-β signaling pathway is the determinant of wound contraction[13,18,19] and a inducer of miR-21 in epithelial cells,[7,9] we investigated the role of miR-21 in the TGF-β–mediated wound contraction using 10T1/2 cells. TGF-β1 induced an obvious increase of miR-21 in 10T1/2 cells as assessed by Northern blot (Figure 4F), which was consistent with our observed increase of miR-21 in granulation tissue. Furthermore, 10T1/2 cells were embedded in collagen I gel to test their contractile capacity by measuring the area of gel block 3 days after TGF-β1 treatment. As seen in Supplemental Figure S4 (available at *http://ajp.amjpathol.org*), miR-21 antagomir could down-regulate the miR-21 level significantly in the 10T1/2 cells in gels. As expected, the 10T1/2 cells with miR-21 knockdown had remarkable reduction of gel contraction compared with control cells (Figure 4G). These results suggest that miR-21 promotes wound contraction on TGF-β stimulation. Although there is a well-defined relationship between miR-21 and proliferation of tumor cells and wound repair cells, the unexpected role of miR-21 in wound contraction is a new finding worthy of further investigation.

Discussion

Wound healing is controlled by a network of biomolecules in a spatiotemporal manner. miRNAs are estimated to regulate the expression of one-third of genes, and their possible roles in regulating wound healing has already been hypothesized[20] and tested.[6–9] Several recent reports have indicated that miRNAs are involved in regulating keratinocyte migration and proliferation during wound healing.[6–9] However, the character of global patterns of miRNAs expression in wound tissue is still unknown. In the present study, we depicted the miRNA profile during granulation tissue formation of wound healing and revealed the important role of up-regulated miR-21 in early wound contraction and collagen deposition during wound healing.

miRNA microarray profiling identified at least 54 miRNAs whose expression changed more than twofold at the stage of granulation formation during wound healing. These miRNAs were closely related to the major events of wound healing, including migration, proliferation, angiogenesis, and matrix remolding. For example, the up-regulation of miR-205, which can promote the migration of keratinocytes,[21] and the down-regulation of miR-200b and miR-429, which prohibits epithelial-mesenchymal transition,[22] suggest that these miRNAs may participate in reepithelialization. The increased expression of miR-31

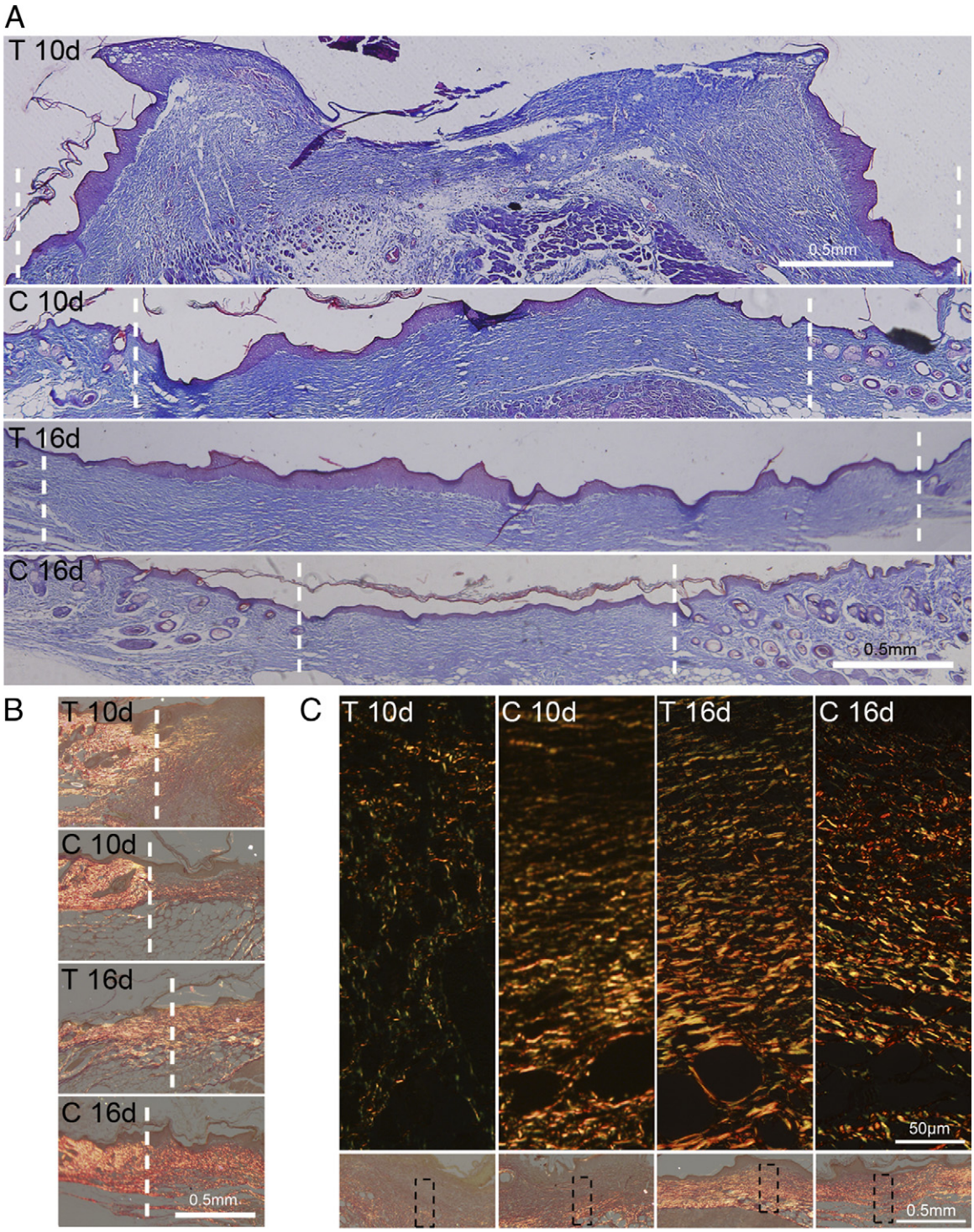

Figure 3. Collagen deposition and remodeling. **A:** The cross-wound sections reveal dense and heavy Masson staining in the dermis with better alignment of collagen fibers in control wound at 10 days and fine collagen network with less staining color at 16 days. The staining pattern of miR-21 antagomir–treated wound at day 16 was similar to that of control wound at day 10. **White dashed lines** indicate the original wound edges. **B:** Comparison of collagen fiber in healthy dermis and granulation tissues in the merged images of bright field and polarized light field. Very strong Sirius red staining is shown in healthy dermis, which clearly defines the unwounded skin. Note that the epithelium and basal lamina are rarely positive for the staining. **C:** The collagen fibers and their alignment were assessed with a polarized light microscope. At day 10 after injury, there is moderate collagen deposition in the form of aligned red short fibers, whereas disordered, shorter, and thinner fibers are seen in green and red in the miR-21 antagomir–treated wounds. At day 16, the collagen fibers in control granulation tissue are matured to form network, but collagen fibers are still in high density in miR-21 antagomir–treated wounds.

and miR-125b also confirmed previous reports that they control the stemness and activation of hair follicle stem cells in wound healing.[23,24] Unexpectedly, the expression of miR-203, an epithelial cell differentiation–related miRNA,[25,26] is enhanced, which may indicate that cell proliferation and differentiation occur simultaneously and the balance between them ensured the progression of healing. The implementation of angiogenesis and circu-

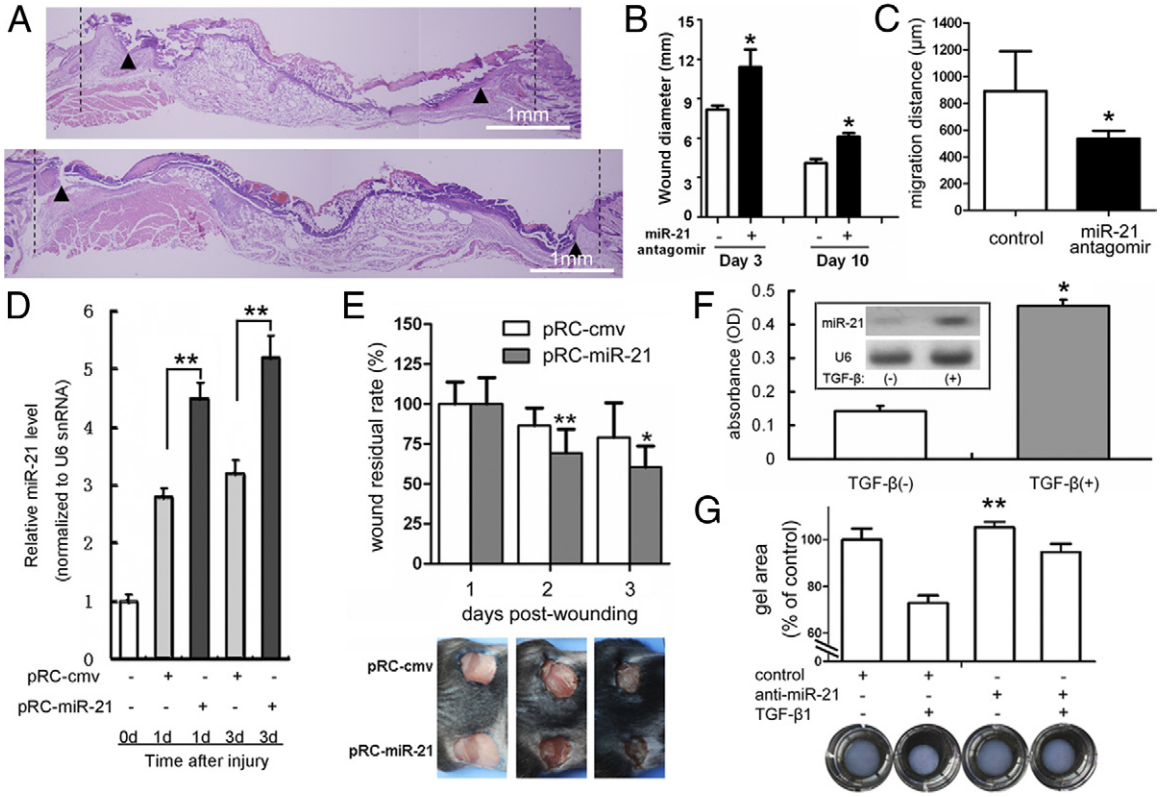

Figure 4. miR-21 regulates early wound contraction. **A:** Local injection of miR-21 antagomir disables early wound contraction. Sections across the center of the wounds 3 days after the injury are shown. **Black dashed lines** mark the border of the original wound edges, and the diameters of wounds are the distances between the two lines. Wound diameter analysis suggests less wound contraction in miR-21 antagomir–treated wounds at 3 days and 10 days after injury (**B**). **Arrowheads** indicate the front point of the migrated epithelial cells, and the migration distance was the distance between the **dashed line** and the corresponding **arrowhead**. The migration distance at 3 days in miR-21 antagomir–treated wounds is significantly shorter than the control (C). $^{*}P < 0.05$ versus control oligos. **D**: Injection of pRC-miR-21 plasmid (20 μg in 200 μL) significantly increased miR-21 expression in wound at 1 day and 3 days after injury ($n = 3$). $^{**}P < 0.01$ versus empty plasmid pRC-cmv treated wound. **E:** Overexpression of miR-21 by injecting pRC-miR-21 plasmid promotes wound contraction. A representative macroview of the wounds at 0, 1, and 3 days after injury (**lower panel**, $n = 3$) and its quantification of wound residual rate (**upper panel**). $^{*}P < 0.05$, $^{**}P < 0.01$ versus pRC-cmv–treated wounds. **F:** When 10T1/2 cells were treated within TGF-β1 (2.5 ng/mL) for 48 hours, miR-21 expression is up-regulated to more than threefold. $^{*}P < 0.05$ versus control vector treatment. **Inset:** Representative result of Northern blot analysis. **G:** 10T1/2 cells treated with miR-21 antagomir are resistant to TGF-β1 (2.5 ng/mL)–induced gel contraction after 3 days of culture. $^{**}P < 0.01$. Data are means ± SD from three independent experiments.

lation may be presented by the up-regulation of angiogenic miR-296,[27] erythropoiesis-related miR-451,[28] and immune response–involved miR-223 and miR-142-5p.[29,30] When comparing the miRNA expression profile of normal skin and 7-day-old wounds, we observed a drastic difference between normal skin and 7-day wounds. Therefore, the interpretation of the microarray data should be cautious. For example, the changes of miR-21 expression may be due to up-regulation or down-regulation at the wound site or to a higher or lower abundance of miR-21–expressing cells. We used the microarray analysis as a primary screen for possible research candidates, although the method itself is unbiased. Furthermore, we verified some of the microarray data, especially for miR-21 by quantitative PCR and *in situ* hybridization, and confirmed that the up-regulation of miR-21 is observed.

Although it has been demonstrated that miR-21 promotes keratinocyte migration in both *in vitro* and *in vivo* models of wound healing,[9] we presumed that miR-21 may regulate wound healing in other ways, such as its involvement in inflammatory reaction, cell proliferation, and matrix remolding in tumors.[31–35] We found that the manifest expression of miR-21 was not only in epithelial cells but also in granulation tissue of the wound by *in situ* hybridization. These results indicated that miR-21 may have multiple roles during wound healing except regulating keratinocyte behaviors.

To identify the role of miR-21 in wound healing, we injected miR-21 antagomir directly to the wound and found that miR-21 inhibition causes significant delay of wound closure and skin reconstruction. A delay of 4 to 5 days in pathologic progression is confirmed in terms of cell proliferation, migration and stratification in the epidermis, and collagen synthesis, deposition, and organization in the dermis. At the very early stage of wound closure (1 to 3 days), the injection of miR-21 antagomir significantly reduces skin tension, which causes the enlargement of the wound. TGF-β is an essential growth factor for wound healing and presents soon after the injury. Our *in vitro* experiments demonstrate that TGF-β–induced mesenchymal phenotype of 10T1/2 cells is accompanied by miR-21 expression and that TGF-β treatment for 3 days induced significant cell gel contraction, which can be blocked by miR-21 antagomir. We believe that miR-21 is also a key regu-

lator of wound contraction, especially at an early stage, in addition to its demonstrated effect in accelerating reepithelialization.[9]

Wound contraction is an ancient and controversial topic.[36–38] One mechanism of wound contraction presumes the cellular force for wound contraction lay on the newly synthesized and highly vascularized wound fibrous connective tissue (ie, granulation tissue).[39] The other mechanism allocates the force to cell migration at the wound margins.[40] Our results from the *in vivo* tests using miR-21 antagomir or expression vector and the *in vitro* contraction of mesenchymal cell–dispersed gel support the second mechanism for early wound contraction because miR-21 takes part in the wound contraction on the first day after the injury, at which time no obvious granulation tissue formation and myofibroblast proliferation are visible. However, at a later stage, the first mechanism may take the primary effect. These *in vivo* and *in vitro* results indicated for the first time that miR-21–driven wound contraction was very important for wound healing. The mechanical force induced by wound contraction may facilitate epidermal cell proliferation, angiogenesis, and migration,[41–43] but the molecular mechanism involved in this process remains to be further investigated. We proposed a model of how miR-21 regulates wound contraction: at the early stage of wound healing (1–3 days), TGF-β and other factors induce miR-21 expression and cause wound margin contraction; when granulation tissue developed under the stimulation of TGF-β and miR-21, the proliferated repair cells and the deposited collagen and other extracellular matrix components may be the main force for wound contraction.

Others have demonstrated that miR-21 is important in fibrosis of the heart, lung, and kidney,[34,35,44] which are featured by excessive deposition of collagen. Liu et al[35] reported that miR-21 promotes collagen deposition by targeting an inhibitory Smad, Smad7. Recently, it is reported that loss of PTEN, a well-known miR-21 target,[45,46] by dermal fibroblasts causes skin fibrosis.[47] These findings are in accord with our finding that miR-21 inhibition resulted in an obvious reduction of collagen in granulation tissue. So, we proposed that miR-21 could be a new target in manipulating tissue regeneration and fibrosis.

In conclusion, the present study has described miRNA profiling of wound healing and demonstrated the involvement of miR-21 in regulating the wound contraction and collagen deposition for the first time. The results suggest that a therapeutic strategy targeting miR-21 expression in wound healing and related disease may be feasible.

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