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Downregulation of miRNAs during Delayed Wound Healing in Diabetes: Role of Dicer

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Delayed wound healing is a major complication associated with diabetes and is a result of a complex interplay among diverse deregulated cellular parameters. Although several genes and pathways have been identified to be mediating impaired wound closure, the role of microRNAs (miRNAs) in these events is not very well understood. Here, we identify an altered miRNA signature in the prolonged inflammatory phase in a wound during diabetes, with increased infiltration of inflammatory cells in the basal layer of the epidermis. Nineteen miRNAs were downregulated in diabetic rat wounds (as compared with normal rat wound, d 7 postwounding) together with inhibited levels of the central miRNA biosynthesis enzyme, Dicer, suggesting that in wounds of diabetic rats, the decreased levels of Dicer are presumably responsible for miRNA downregulation. Compared with unwounded skin, Dicer levels were significantly upregulated 12 d postwounding in normal rats, and this result was notably absent in diabetic rats that showed impaired wound closure. In a wound-healing specific quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) array, 10 genes were significantly altered in the diabetic rat wound and included growth factors and collagens. Network analyses demonstrated significant interactions and correlations between the miRNA predicted targets (regulators) and the 10 wound-healing specific genes, suggesting altered miRNAs might fine-tune the levels of these genes that determine wound closure. Dicer inhibition prevented HaCaT cell migration and affected wound closure. Altered levels of Dicer and miRNAs are critical during delayed wound closure and offer promising targets to address the issue of impaired wound healing.

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INTRODUCTION

Wound healing is a complex biological process involving an intricate crosstalk of cellular events including inflammation, reepithelialization and restoration of affected tissues (1,2). All these events are controlled by diverse growth factors, cytokines, matrix proteins and a variety of cell types. Homeostasis initiates the first response during wound healing, and this leads to movement of several cytokines, chemokines and growth factors (such as platelet-derived growth factor [*Pdgf*] and transforming growth factor β [*Tgfβ*]) toward the wound site. Invasion of neutrophils into the wound area primarily removes the resident microbes, and this step is followed by active movement of macrophages and monocytes that act to remove dead tissues and other debris materials (3,4). These phenomena involve the generation of reactive oxygen species and nitric oxide, and these inflammatory events are followed closely by reepithelialization that is achieved by migration and proliferation of keratinocytes and fibroblasts in and around the wound site. Also, epidermal cells undergo desmosomal dissolution and formation of peripheral cytoplasmic actin filaments. Subsequently, basement membrane proteins orderly reappear and epidermal cells tend to get attached to the basement membrane and dermis followed by formation of granulation tissue (1,5).

Any interference during these orchestrated events of wound healing leads to delayed wound healing, which is major cause of gangrene and limb amputations (6,7). Diabetes is a major cause of delayed wound healing, and various factors have been reported to be associated with this complication. Local ischemia due to vascular damage during diabetes significantly contributes to a delay in wound closure. Damage to the extracellular matrix (ECM), impaired granulocyte function and movement, altered neovascularization, increased proteinase activity and defective macrophage function all contribute to delayed wound healing during diabetes (8–11). While a lot of studies report the involvement of several genes and pathways responsible for this complication, little is known of the role of microRNAs (miRNAs) in this phenomenon.

miRNAs are a class of endogenously expressed noncoding RNAs of ~22 nucleotides in length that regulate gene

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expression primarily at the posttranscriptional level by translational repression or mRNA degradation/mRNA destabilization (12,13). They have been implicated in various physiological processes and pathological states, and altered levels of miRNAs have been reported in several diseases (14–16). In wound healing, too, few studies demonstrate the roles of miRNAs (17,18) and deregulated miRNA signatures are associated with impaired wound healing. miR-210 that is induced under hypoxic conditions targets *E2f3* and inhibits keratinocyte proliferation during wound closure (19). Induction of miR-200b during wounding suppresses angiogenesis by targeting globin transcription factor 2 (*Gata2*) and vascular endothelial growth factor receptor 2 (*Vefgfr2*) (20). Several miRNAs including miR-130a and miR-21 are aberrantly expressed in venous ulcers, and they delay epithelization in an acute human skin wound model (21). miR-146a is downregulated in wounds of diabetic mice and correlates with the expression of inflammatory genes (22), thereby delaying the process of wound closure. In type 2 diabetic KKAy mice, 14 miRNAs were differentially regulated in the wounded skin, and miR-21 was identified to be critical in fibroblast migration (23). In chronic nonhealing diabetic ulcers, persistent elevated miR-198 expression impairs keratinocyte migration and reepithelialization, thereby delaying wound closure (24). Except for these sparse reports, not much has been reported on the roles and mechanisms of miRNAs in delayed wound healing during diabetes. Prolonged inflammation is one of the critical factors for delayed wound closure. A normal inflammatory phase during wound closure peaks at ~1–4 d before moving into the next phase of granulation and proliferation. In a diabetic wound, the process many times is stuck in the inflammatory phase because of a variety of altered factors that prolong this phase and prevents the initiation of the next phase, thereby delaying the wound-healing process (25,26). Therefore, several studies are aimed at identifying processes that prolong the inflammatory phase.

In this study, we sought to interrogate miRNA signatures in the diabetic rat wound that is stuck in the inflammatory stage compared with the normal rat wound that shows a normal healing pattern. We report an altered miRNA signature in the wounded skin of diabetic rats during the prolonged inflammatory phase and present interacting networks of their predicted targets with specific wound-healing genes.

MATERIALS AND METHODS

Animals and Induction of Diabetes

The study was approved by the Institutional Animal Ethical Committee. Male Sprague Dawley rats aged 10–11 wks (225–275 g) were procured from the National Institute of Nutrition (Hyderabad, India). Animals were allowed to acclimatize for 2 wks under a 12:12 light–dark cycle and were given food and water ad libitum throughout the experiment. Ten days before wounding, rats received intraperitoneal injection of streptozotocin (65 mg/kg body weight in citrate buffer 0.1 mol/L, pH 4.5.) to induce diabetes (n = 6 in each group). Control rats were injected with citrate buffer. On the third day after injection, blood glucose levels were measured by tail vein bleeding (Accu-Chek Glucometer). Rats with persistent blood glucose levels ≥250 mg/dL until wounding were confirmed diabetic and used for the experiment. Control rats had blood glucose levels in the range of 90–110 mg/dL. All animals were weighed at regular intervals and observed for side effects. While the control (normal) rats gained weight until the end of the experiment (245 ± 3 g [before] and 283 ± 4 g [end of the experiment]), there was a significant decrease in the body weights of streptozotocin-induced diabetic rats (269 ± 3 g [before] and 153 ± 7 g [end of the experiment]). As compared with normal rats, the daily food and water intake increased in diabetic rats.

Creation of Wounds

Ten days after induction of diabetes, two full thickness excisional wounds with splints were created on the dorsal surface of the animals (n = 6 in each group). The rat's dorsal surface hair was first trimmed and depilated 24 h before wounding. During the entire wounding procedure, rats were anesthetized with isofluorane (4% in 100% oxygen at a flow rate 1 L/min) and anesthesia was maintained using 1–3% isofluorane. Dorsal skin was wiped with an alcohol swab and 10% povidone-iodine solution was applied. Two symmetrical wounds were made on the dorsum of the rats on either side of the spine by using a sterile 5.0-mm diameter punch biopsy tool (Dermaindia). Silicone splints were placed around the wound area, and anchoring of the splint was done with four nylon sutures to ensure positioning. Immediately after splinting, wounds were dressed (Tegaderm, 3M). Rats were housed individually after the procedure to preserve skin and dressing integrity. On d 1, 7 and 12 postwounding (corresponding to normal phases of inflammation, granulation/proliferation and matrix remodeling, respectively), animals were euthanized by a single intraperitoneal injection of sodium thiopentone (Neon Laboratories Limited), and wounded tissue together with adjoining tissues of ~0.5 mm were excised. Samples from normal and diabetic skin (unwounded [isolated on the same day as d-7–postwounding tissue] and wounded [d 1, 7 and 12 postwounding]) were washed in 1 × phosphate-buffered saline (PBS) and stored in –80°C for protein estimation or collected in RNA later solution (Ambion) for RNA isolation. Tissues in RNA later were kept at 4°C overnight and then frozen at –80°C until RNA isolation. Another set of wound tissue was collected from normal and diabetic rats (d 7 postwounding) and preserved in formaldehyde for hematoxylin–eosin (H&E) staining.

H&E Staining of the Wounded Tissue

Formaldehyde-preserved wounded tissues from normal and diabetic rats (n = 6) were paraffin-embedded and cut at a thickness of 5 μm for H&E staining. Stained sections were then examined by light microscopy (20×, Eclipse 80*i*, Nikon),

and images were acquired using the NIS-elements imaging software. Quantitative analysis of the inflammatory cells in normal and diabetic sections was done by dividing the stained sections into equal number of grids and counting the inflammatory cells in the same number of grids within the same region. Results are presented as number of inflammatory cells per mm^2 of area.

RNA Isolation and miRNA Array

miRNA array was done in normal and diabetic rat skin and also in wounds of normal and diabetic rats (n = 6) (d 7 postwounding). Collection of unwounded skin tissues from normal and diabetic rats was done on the same day as that of wounds collected from normal and diabetic rats (d 7 postwounding). Total RNA (from normal skin, diabetic skin, normal skin wound and diabetic skin wound) was isolated by using the TRIzol reagent (Ambion, Life Technologies) according to the manufacturer's protocol. The quality and yields of RNA were analyzed by agarose gel electrophoresis and quantified by using Nanodrop 2000 (Thermo Fisher Scientific). miRNA expression was evaluated with the TaqMan Array Rodent MicroRNA card A v2.0 (Applied Biosystems). RNA (500 ng) was reverse-transcribed with the Taqman MicroRNA Reverse Transcription Kit and Megaplex RT Primers (Rodent Megaplex RT Primers Pool A, Applied Biosystems). The cDNA was combined with a Universal PCR Master Mix, NoAmpErase UNG (Applied Biosystems) and loaded into the arrays. TaqMan real-time polymerase chain reaction (PCR) was performed in a 7900 HT Fast Real-Time PCR system (Applied Biosystems) according to the manufacturer's protocol. Data were analyzed with the $2^{-\Delta\Delta Ct}$ method, where the average Ct (ΔCt) is the normalized Ct values of the miRNA with that of U87 (reference control) and miRNAs altered in (a) unwounded diabetic rat skin compared with normal rat skin and (b) diabetic skin wound compared with normal skin wound in rats (d 7 postwounding), with a p value <0.05 (compared with the respective normal rats) considered as statistically significant.

Wound-Healing PCR Array

Gene expression profiling for genes specific to the wound-healing process was done using the Wound Healing RT^2 Profiler PCR Array (SABiosciences). A total of 3 μg RNA (from normal rat skin, diabetic rat skin, normal rat skin wound or diabetic rat skin wound) of each normal and diabetic rat (n = 6) were reverse-transcribed, and the cDNA was subjected to PCR on a LightCycler 480 Real-Time PCR System (Roche) by using the Wound Healing RT^2 Profiler PCR Array according to the manufacturer's instructions. Expression data generated was background corrected and normalized to the built-in housekeeping genes' panel by using the Web-based PCR array data analysis software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) available from SA Biosciences. Here, the relative amounts of transcripts in the diabetic skin compared with the normal rat skin or those in the diabetic rat skin wound compared with normal rat skin wound (d 7 postwounding) are calculated by the $\Delta\Delta$Ct method. Fold changes with a p value <0.05 were taken as statistically significant.

To assess the transcript levels of Dicer in the normal and diabetic rat tissue (unwounded and wounded [d 1, 7 and 12 postwounding]), cDNA as prepared above was subjected to real-time PCR by using Dicer specific primers (forward 5′-TTCCA GAGTG CTTGA GGGAG-3′ and reverse 5′-CGCCT GAAGT TGAGC TCATC-3′). SYBR Green PCR Master Mix (Applied Biosystems) was used according to the manufacturer's instructions (Applied Biosystems). Data were analyzed as described by Pfaffl (27), and results are expressed as the fold change (with respect to normal unwounded rat skin) in Dicer expression after normalization to 18S rRNA.

Western Blot

Rat skin (normal and diabetic, collected on the same day as wounds on d 7 postwounding) and skin wound tissues (d 1, 7 and 12 postwounding, n = 4) were lysed in RIPA lysis buffer containing protease inhibitors (Calbiochem). Protein samples (90 μg) were resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting by using an anti-Dicer antibody (Santa Cruz Biotechnology). Immunoreactive bands were detected by using the ECIL Western Blotting Kit (Thermo Fisher Scientific). Hsc70 (Santa Cruz Biotechnology) was used as a loading control.

Generation of Networks

We sought to assess interactions between the altered wound-healing genes and the predicted targets to the differentially altered miRNAs. Potential regulators enriched for the altered wound-healing genes were extracted ($p < 0.05$) from ChEA within the ENRICHR tool (http://amp.pharm.mssm.edu/Enrichr/). Targets to the altered miRNAs were identified by TargetScan (http://www.targetscan.org/index.html). Of this list of potential miRNA-predicted targets, only those targets were prioritized that were also regulators to the altered wound-healing genes (from CHEA). Networks were generated with these regulators and the altered wound-healing genes using Cytoscape v3.0.2 (28) with nodes (as genes) and edges with a flat arbitrary numerical value of 1.0.

Transfection and Quantitative Reverse Transcriptase (RT)-PCR

HaCaT cells were plated in six-well plates and allowed to grow to 40% confluence in Dulbecco's modified Eagle's medium (DMEM) F12 media (Sigma) supplemented with 10% fetal bovine serum in an atmosphere of 5% CO_2 at 37°C. They were transfected with either the scramble or Dicer siRNA (10–100 nmol/L; Dharmacon) by using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). After 24 h, cells were collected and RNA was isolated as described above.

A total of 2 μg RNA from each sample was reverse-transcribed, and the cDNA was subjected to real-time PCR as described above with human specific Dicer primers (sense CAGAC TGTCG TGCCG TATTG and antisense ATTTC CTGTG CAGCT CCTCT). Also, protein levels of Dicer were evaluated by Western blot analyses in the absence and presence of Dicer siRNA (10–100 nmol/L) as described above. Lamin (Santa Cruz Biotechnology) was taken as the loading control.

Wound-Healing Assay

In scramble and Dicer siRNA (10–100 nmol/L) transfected HaCaT cells, a wound was made with a sterile 10-μL pipette tip, such that cells along the wound are scrapped off and a sharp visible line at the wound edge is left. Cells were washed with PBS to remove cell debris, and the culture medium was replaced with DMEM-F12 media. After 6, 12 and 24 h, cells were examined and photographed under a phase-contrast microscope (10×, Ti-S, NIKON). The time-dependent movement of cells into the wound area at 6, 12 and 24 h were monitored and quantified using the T-Scratch software (http://cse-lab.ethz.ch/software).

Densitometric Analysis

For densitometric analyses of the Western blots, the Alpha DigiDoc 1201 software (Alpha Innotech Corporation) was used to evaluate protein expression.

Statistical Analysis

All bars represent mean ± standard error of the mean (SEM) or standard deviation (SD), and data were analyzed using a Student *t* test. *p* Values <0.05 were considered statistically significant. Correlations between miRNA levels and decreased Dicer levels were evaluated using the online tool found at http://www.alcula.com/calculators/statistics/correlation-coefficient, and *p* values were calculated (http://www.danielsoper.com/statcalc3/calc.aspx?id = 44).

RESULTS

Delayed Wound Closure during Diabetes

Wounds (5 mm) were created on the dorsal skin of normal and streptozotocin-induced diabetic rats, and the progress of wound closure was followed until the 12th day of wounding. After 7 d of wounding, while wounds of normal rats showed marked visible closure, the diabetic wound was significantly larger with negligible signs of healing. On the 12th day postwounding, normal wounds were completely healed, while wounds in diabetic rats were still open and showed substantial delay in healing (Figure 1A). Wounds in normal skin showed signs of healing starting from d 3 onward that improved further on subsequent days; however, wounds in diabetic skin showed limited progress toward closure of the wound. The wound area in both cases was quantified, and the relative values are shown in Figure 1B. Wound healing during diabetes is characterized by a prolonged inflammatory phase that interferes with the timely initiation of subsequent phases, thereby delaying wound closure. We evaluated the status of inflammation in the normal and diabetic rat skin wounded tissue on d 7 postwounding. In the diabetic rat wound, there was increased presence of inflammatory cells in the basal membrane of the epidermis. In the normal, nondiabetic wound, few inflammatory cells were visible, and this was associated by an almost complete closure of the wound (Figure 1C). The status of inflammatory cells was quantified, and the relative number of inflammatory cells in normal and diabetic rat skin wound (d 7 postwounding) is shown in Figure 1D. Compared with the normal wound, there was more than a three-fold increase in the number of inflammatory cells in the wounds of diabetic rat skin.

miRNAs of the Skin Are Downregulated in a Diabetic Rat Wound

In this study, we sought to interrogate the miRNA signature in the wounds of diabetic rats at a stage when it is stuck in the prolonged inflammatory stage versus the normal wound that undergoes an orchestrated normal healing process. As described by Grice *et al.* (29), before attempting to evaluate the altered miRNA signature in the rat wounded tissue during diabetes, we first compared the status of miRNAs in normal and diabetic rat unwounded skin using the Taqman Array Rodent miRNA card (Applied Biosystems) to set a baseline for alterations as a result of wounding. As compared with the normal rat skin, 10 miRNAs were differentially expressed in the skin of diabetic rats; two were upregulated and eight were downregulated (Figure 2A). A volcano plot of the differentially regulated miRNAs in the diabetic rat unwounded skin compared with the normal rat unwounded skin is shown in Figure 2B, which shows upregulated and downregulated miRNAs in the diabetic skin (as compared with normal skin), among which 10 were significantly altered ($p < 0.05$) as stated above. Subsequently, miRNA expression was evaluated in normal and diabetic skin wounds in rats (d 7 postwounding). Altered miRNAs together with their fold changes (with respect to the normal rat skin wound, d 7) are shown in Figure 2C, and the volcano plot is shown in Figure 2D. Compared with the wounded skin of normal rats on d 7 postwounding, 19 miRNAs were downregulated (none upregulated) in the wounds of diabetic rats at $p < 0.05$, suggesting downregulation of miRNAs in the wounds of these diabetic rats. The volcano plot (Figure 2D) also shows a tendency of almost all miRNAs toward getting inhibited in the wounds of diabetic rats compared with those of normal rats on d 7 postwounding. However, this is in contrast to the miRNA signature in the diabetic rat unwounded skin (compared with the normal rat skin), where miRNAs are both upregulated and downregulated (Figures 2A, B). From among the downregulated set of miRNAs in wounded skin of diabetic rats (compared with the wounds of normal rat skin), three miRNAs (miR-31, miR-497 and miR-125b-5p) were also downregulated

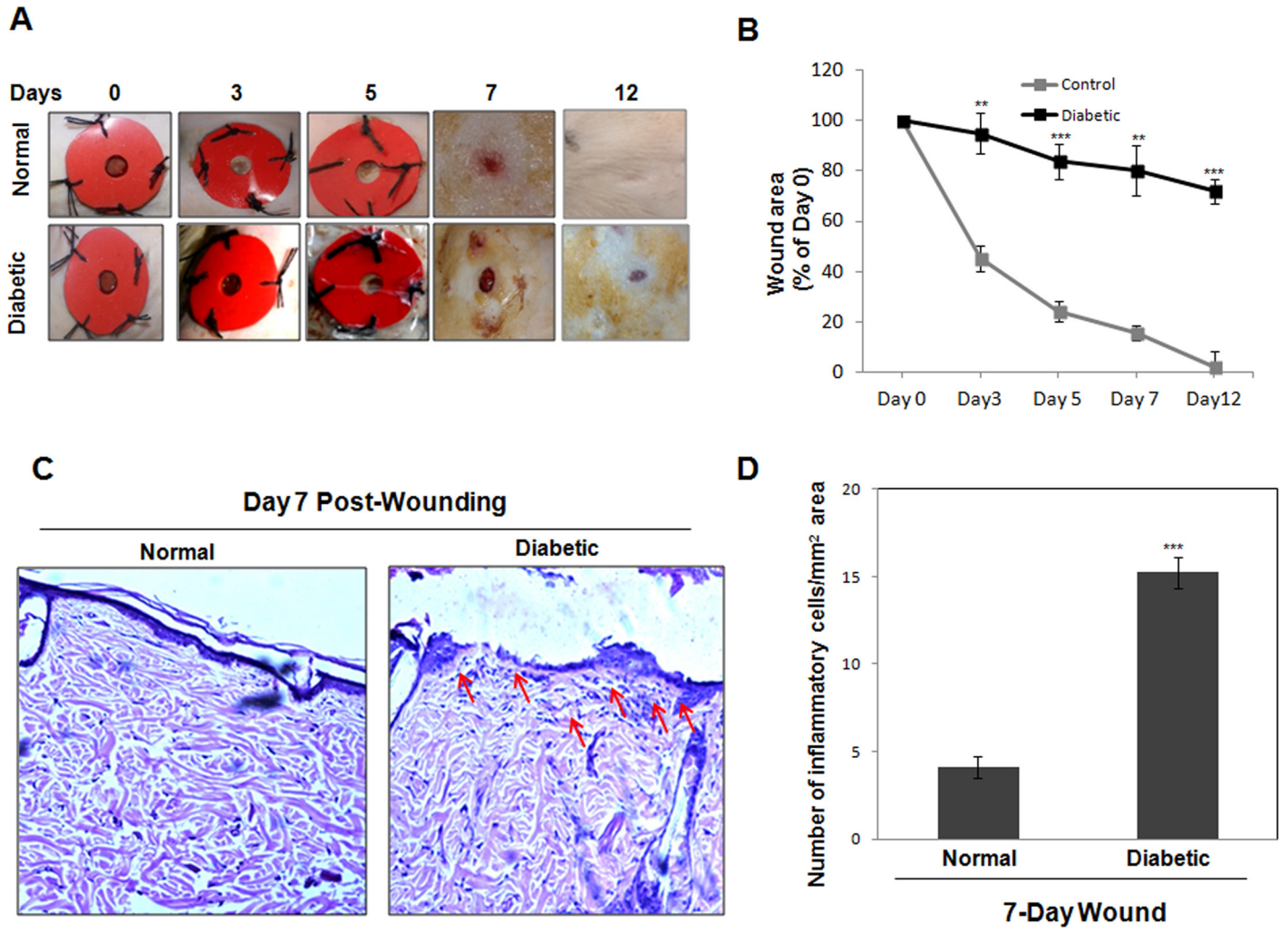

Figure 1. Delayed wound healing in diabetic rats. (A) Two 5.0-mm wounds were created on the dorsal surface in normal and streptozotocin-induced diabetic rats (n = 6), and the progress of wound closure was followed until 12 d postwounding. (B) The wound area in both groups of animals was measured at each time point and the mean values are presented. Each point is the mean ± SEM of six animals. (C) Normal and diabetic wounded skin tissues (d 7 postwounding) were paraffin-embedded and sections (5 μm) were stained with H&E. Presence of inflammatory cells in the wounded diabetic skin is shown by red arrows (magnification 20x). (D) Inflammatory cells in both normal and diabetic wound (d 7 postwounding) were quantified as described in Materials and Methods, and results are presented as the number of inflammatory cells per mm^2 area. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with wounds in normal animals on the same day.

in the unwounded diabetic rat skin (compared with unwounded normal rat skin), although their fold inhibition was more pronounced in the wounded tissue during diabetes (compared with normal rat wound).

Since the RNase III enzyme, Dicer, catalyzes the formation of mature miRNAs and we observed an inhibition of miRNAs in diabetic wounds in rats (d 7 postwounding), we evaluated the status of Dicer in normal and diabetic unwounded and wounded rat skin. Dicer is necessary for the biogenesis of most miRNAs. It is a cytosolic enzyme that cleaves pre-microRNA (pre-miRNA) into short mature miRNA fragments. As shown in Figure 3A, on d 7 postwounding, compared with wounds in normal rats, there was a significant decrease in the transcript levels of Dicer in diabetic wounds, suggesting that this might be responsible for the inhibition of miRNAs (Figures 2C, D). Also on d 12 postwounding, Dicer levels remained inhibited in the diabetic rat wounds (compared with normal rat wounds). A modest but insignificant alteration in Dicer levels was observed in the diabetic skin tissue compared with normal skin and also in diabetic skin wound on d 1 postwounding (compared with normal skin wound). Interestingly, in normal rats, there was a significant increase in Dicer levels on d 12 postwounding (compared with normal rat unwounded

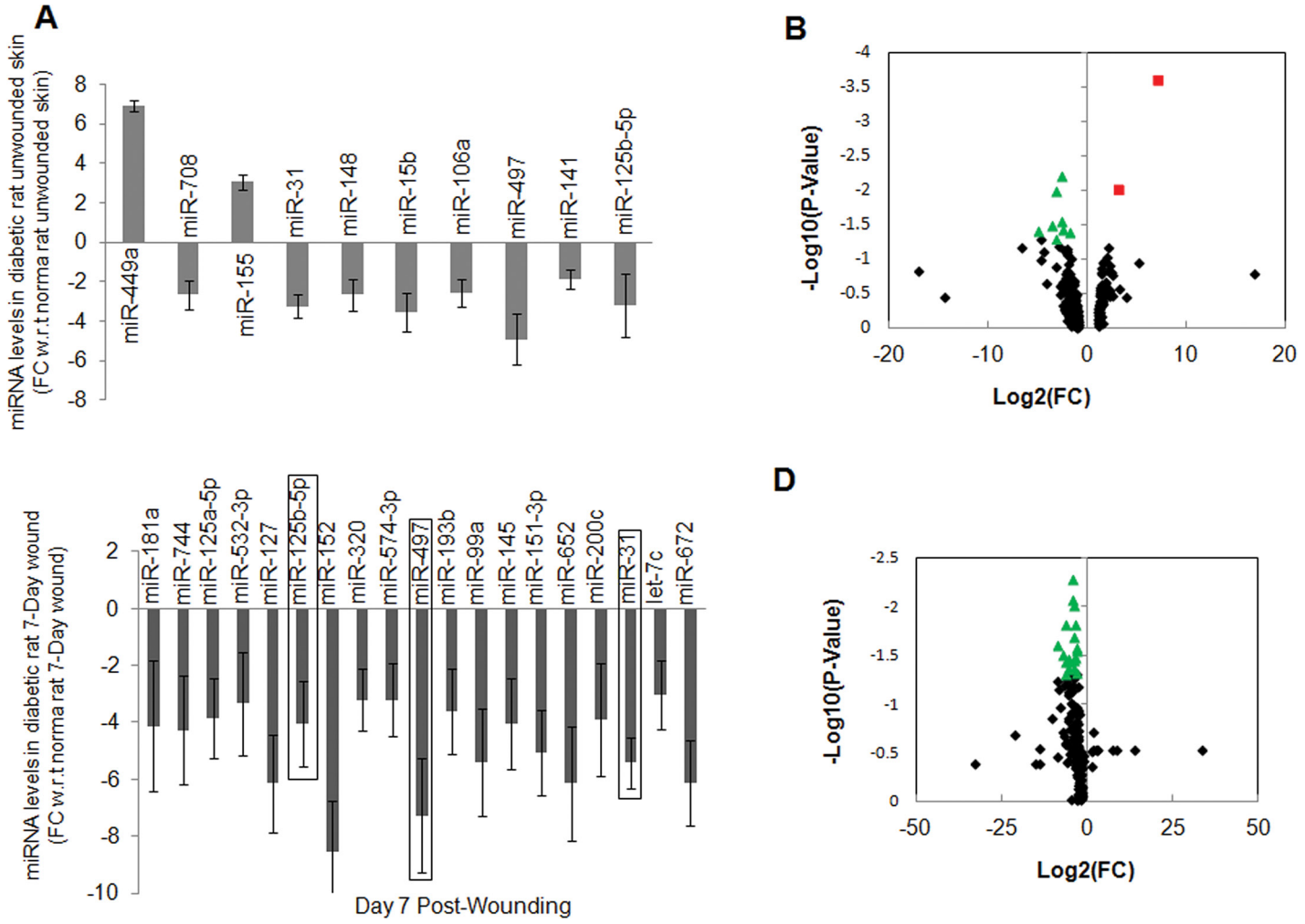

Figure 2. Downregulation of miRNAs in wounds of diabetic rats on d 7 postwounding. (A) Total RNA was isolated from normal and diabetic rat skin (unwounded) (n = 6, isolated on the same day as collection of wound tissues on d 7 postwounding), 500 ng RNA was reverse-transcribed and quantitative PCR was done on a TaqMan Rodent miRNA card, as described in Materials and Methods. The significantly ($p < 0.05$) altered miRNAs (in the diabetic rat skin compared with the normal rat skin) along with their fold changes are shown. (B) In the volcano plot of the above RT-PCR array, the green triangles are the downregulated miRNAs and the red squares are the upregulated miRNAs. (C) Total RNA was isolated from wounds of normal and diabetic rats (n = 6) on the seventh day postwounding and reverse-transcribed (500 ng), and quantitative PCR for miRNAs was done as in (A). miRNAs altered in the wounds of diabetic rat skin (compared with normal rat wound on d 7 postwounding) together with their fold changes is shown and the volcano plot for the experiment is given in (D) (downregulated miRNAs are green triangles). miRNAs enclosed within black rectangular boxes (in C) are common to those altered in the diabetic unwounded skin (compared with normal rat unwounded skin, A). All miRNAs in (A) and (C) are significantly altered with $p \leq 0.05$ and values are means ± SD. FC, fold change; w.r.t., with respect to.

skin), suggesting that an increase in Dicer levels is possibly associated with wound closure in normal rats. Protein levels of Dicer were also evaluated in the unwounded skin (diabetic rats compared with normal rats) and on d 1, 7 and 12 postwounding (diabetic rat wounds compared with normal rat wounds). As in the transcript levels, Dicer protein levels significantly decreased on d 7 and 12 postwounding in the diabetic skin (compared with normal rat skin wounds on the respective days postwounding) (Figure 3B). Dicer levels did not change either in the unwounded diabetic skin or on d 1 or 7 postwounding in normal rats (compared with unwounded normal rat skin). Dicer protein levels depicted a significant increase in the normal rats on d 12 postwounding compared with the unwounded skin.

Having observed a decrease in miRNA and Dicer levels in the wounds of diabetic rat skin (compared with normal rat wounds on d 7 postwounding), we sought to evaluate the correlation coefficient between decreased miRNA levels and

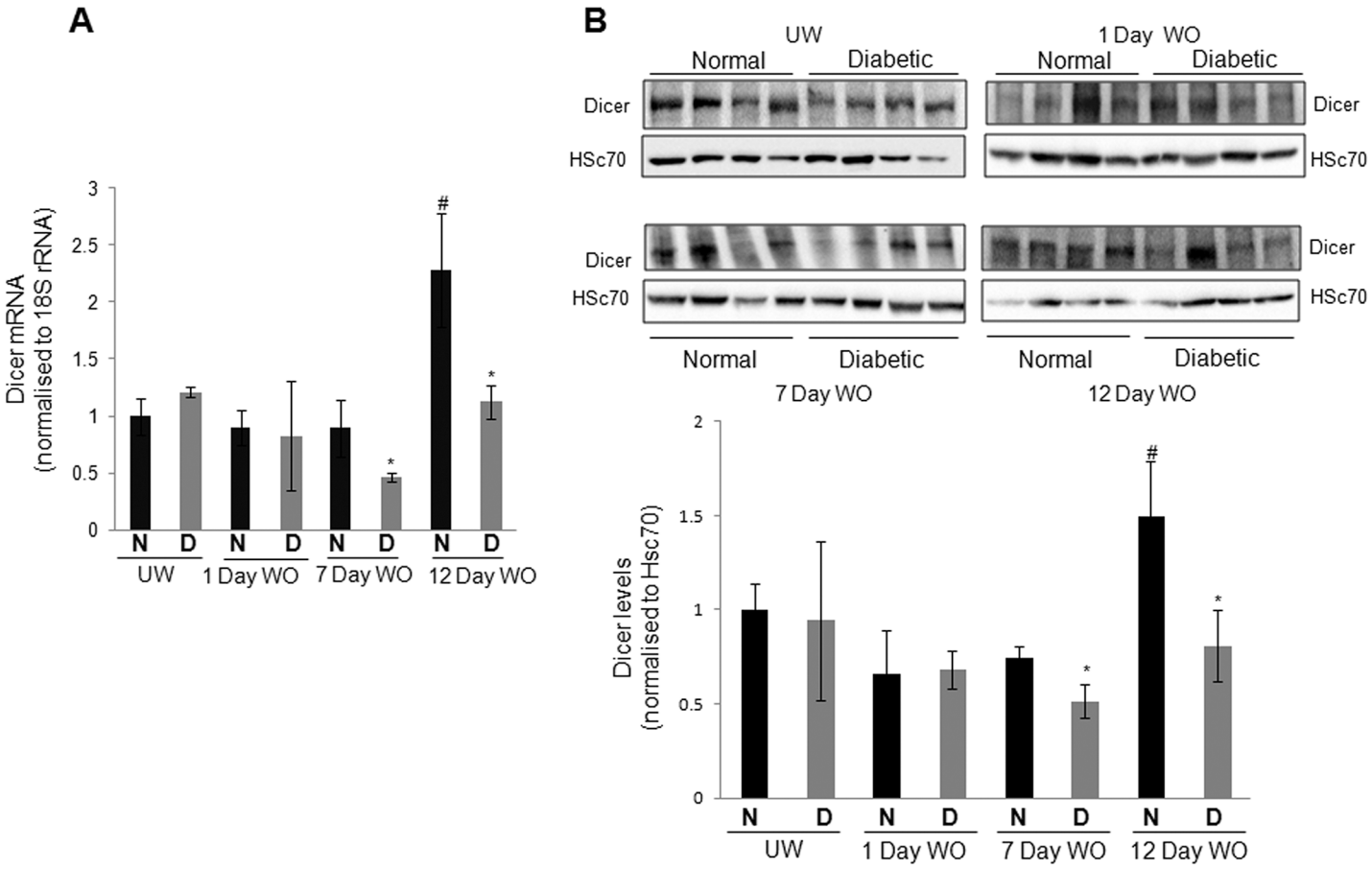

Figure 3. Dicer levels are downregulated in the diabetic rat skin wounds on d 7 and 12 postwounding. (A) Total RNA from normal and diabetic unwounded (UW) and wounded (WO) (d 1, 7 and 12 postwounding) rat skin (2 μg) was reverse-transcribed, and levels of Dicer were quantified by quantitative RT-PCR (qRT-PCR) using specific primers. Data were normalized to 18S rRNA (n = 6). Transcript levels of Dicer in each sample with respect to that in the normal unwounded rat skin are shown. (B) Skin samples as in (A) were collected and lysed, and lysates (90 μg) were subjected to Western blot analyses using anti-Dicer antibody (n = 4). Hsc70 was taken as the loading control. Densitometric analyses are provided in the panel below the Western blots, and results at each stage are Dicer levels as compared with normal rat skin (unwounded). UW, unwounded; WO, wounded. All values are means ± SEM. $^{*}p < 0.05$ compared with normal wound tissues on the respective day postwounding. $^{\#}p < 0.05$ compared with normal rat skin (unwounded).

inhibited Dicer levels. Decreased miRNA levels showed positive correlation with decreased Dicer levels (*r* values ranging from 0.6 to 0.94 for all miRNAs), with several of them at a significant *p* value ≤0.05.

Wound-Healing Genes Are Altered in the Wounds of Diabetic Rats

To specifically evaluate the status of genes involved during the wound-healing process during diabetes, RNA from the wounded tissue of normal and diabetic rats (d 7 postwounding) were subjected to PCR analysis on a wound-healing RT^2 Profiler PCR array (n = 6). Before this, we first compared basal levels of these genes in the normal and diabetic rat skin (unwounded). As shown in Figure 4A, eight genes were significantly ($p < 0.05$) altered in the diabetic rat skin (unwounded) compared with the skin of normal rats. Collagen type 1α2 (*Col1a2*) and collagen type 1α1 (*Col1a1*) were significantly decreased in the skin of diabetic rats. Diabetes is accompanied by decreased collagen content in the skin that is associated with the progressive decline in the integrity of the skin during diabetes. vascular endothelial growth factor alpha (*Vegfα*) levels were also decreased, and this is a major factor for decreased angiogenesis and delayed wound closure (30). Genes such as *Egf* and *Ctsl1* were upregulated. *Ctsl1* is essential for the development of type 1 diabetes in NOD mice, and its deficiency protects mice from insulitis and diabetes (31). *Egf* belongs to a family of growth factors that regulate cell proliferation and migration by binding to epidermal growth factor receptor (*Egfr*) on the cell surface. Increased phosphorylated–epidermal growth factor receptor (p-*Egfr*) has been associated with the skin during diabetes in an oxidative stress-prone environment (32). In a similar

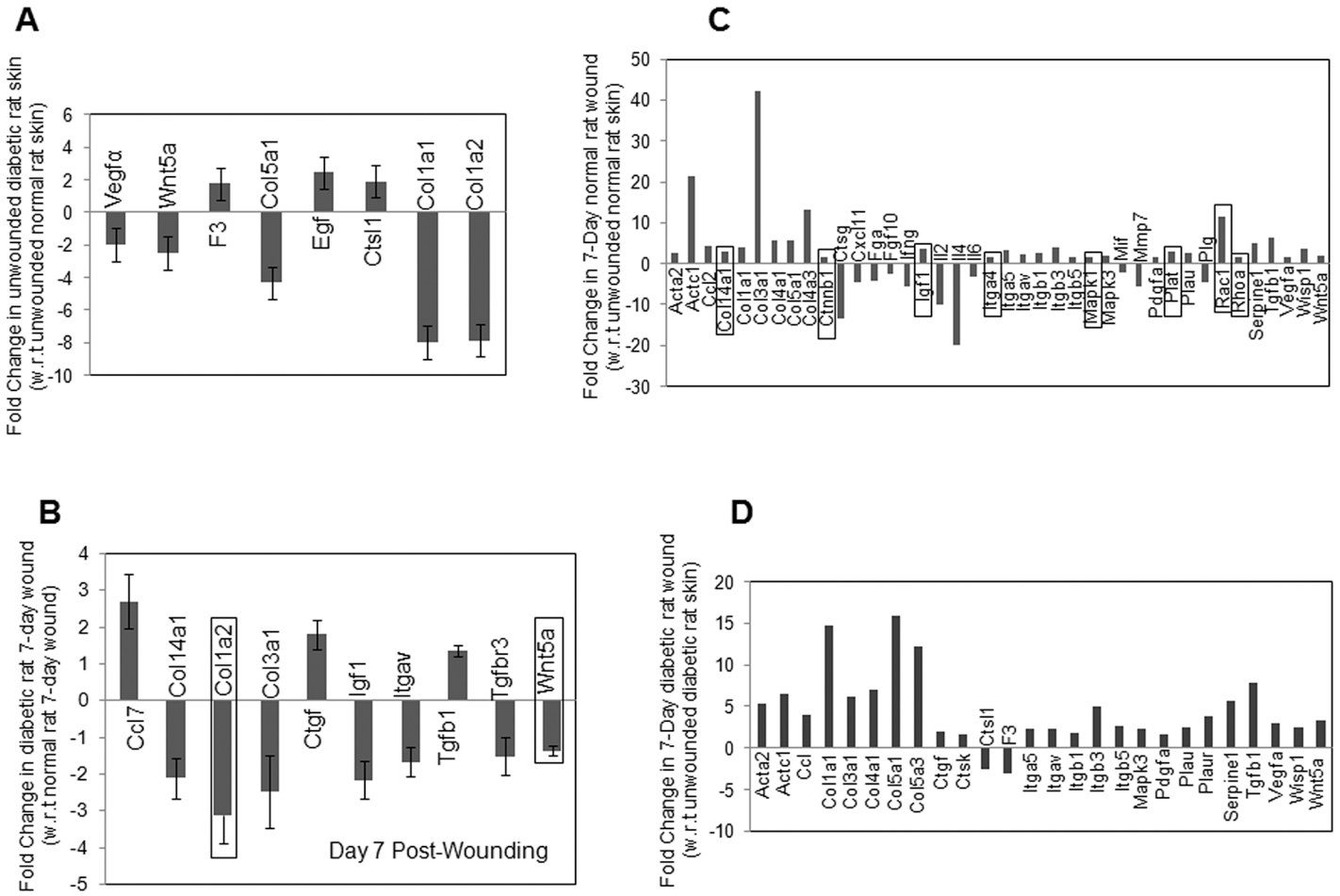

Figure 4. Wound-healing genes are differentially regulated in diabetic (unwounded and wounded) skin (A). Skin tissues (unwounded) were collected from normal and diabetic rats on the same day as wound tissues (d 7 postwounding). Total RNA was isolated and 3 μg total RNA was reverse-transcribed, and cDNA was used to evaluate the status of specific wound-healing genes using a Wound Healing RT^2 profiler PCR Array, as described in Materials and Methods. The significantly ($p < 0.05$) altered wound-healing specific genes in the diabetic rat skin compared with the normal rat skin (unwounded) are shown with their respective fold changes. (B) Total RNA (3 μg) from wounds of normal and diabetic rats (d 7 postwounding) was reverse-transcribed and subjected to PCR using the Wound Healing RT^2 profiler PCR Array, as in (A). The differentially regulated genes ($p < 0.05$) in the diabetic rat wound (compared with the normal rat wound) with their fold changes is shown. Genes that are altered, both in the unwounded diabetic skin (as compared with normal rat skin, as in A) and wounded diabetic skin (as compared with wounded normal rat skin, d 7 postwounding), are enclosed by black rectangular boxes in (B). (C) Differentially expressed transcript levels of wound-healing specific genes in normal rat wound compared with normal rat unwounded skin are presented. A total of 40 genes were differentially expressed ($p < 0.05$) in the normal rat wound during the healing process (compared with normal rat unwounded skin). (D) Transcript levels of wound-healing genes in diabetic rat wound compared with diabetic rat skin (unwounded). All genes shown were differentially expressed in the diabetic rat wound (d 7 postwounding) with a p value <0.05. Compared with altered genes in (D), wound-healing genes that were upregulated only in the normal rats during the healing process (as in C) are marked in black rectangular boxes in (C). The experiment was done in six animals each from the normal and the diabetic groups. Values are means ± SD.

transcriptome study in normal and diabetic db/db mice skin, transcripts upregulated during diabetes were overrepresented in categories of lipid metabolism and wounding, whereas the downregulated genes majorly mapped onto developmental processes (29).

In the diabetic rat wounded tissue on d 7 postwounding, 10 genes were significantly altered ($p < 0.05$) compared with normal rat wounded tissue (d 7 postwounding) (Figure 4B). In addition to the collagens that were altered in the diabetic rat skin, other members of the collagen family, namely, collagen type XIV α1 (*Col14a1*) and collagen type 3α1 (*Col3a1*) were significantly downregulated, suggesting additional decrease of collagen levels in wounds of diabetic rats. Wingless type mouse mammary tumor virus (MMTV)

integration site family member 5A (*Wnt5a*), transforming growth factor β receptor 3 (*Tgfβr3*), insulin-like growth factor 1 (*Igf1*) and integrin α V (*Itgav*) were also markedly downregulated. Integrins and insulinlike growth factors are extremely critical during the wound-healing process, and their inhibition interferes with the normal process of wound healing and delays the same during diabetes (40). Three genes, namely *Ccl7*, *Ctgf* and *Tgfβ1*, were upregulated. *Ccl7* is a chemokine specifically responsible for attracting monocytes and regulating macrophage function. Its upregulation in a diabetic wound presumably is an adaptive event to facilitate wound closure. Connective tissue growth factor (*Ctgf*) and *Tgfβ1* are growth factors that promote migration, proliferation and angiogenesis; however, excessive and prolonged increases in these factors have been suggested to be antagonistic to the wound-healing process, and transgenic mice constitutively overexpressing *Tgfβ1* exhibit significant delay in full thickness wound healing compared with nontransgenic mice (33).

In addition, we also evaluated the wound-healing genes' signature during the healing process in normal rats (d 7 postwounding versus unwounded) to follow the pattern of genes' behavior as the wounds heal during a normal healing process. As shown in Figure 4C, 40 genes were altered in the normal rat wound on d 7 postwounding compared with the unwounded normal rat skin. A total of 11 genes were downregulated, and 29 genes were upregulated. An identical analysis of the status of wound-healing genes in the diabetic rat wound (d 7 postwounding) versus the unwounded diabetic skin identified 34 genes to be differentially expressed compared with the unwounded diabetic rat skin (Figure 4D). Eight genes (namely, *Rac1*, *Col4a1*, *Igf1*, *Plat*, *Mapk1*, *Ctnnb1*, *Itga4* and *Rhoa*) were significantly ($p < 0.05$) upregulated during the healing process in normal rats (Figure 4C) but were not altered in the diabetic rats (Figure 4D), suggesting that upregulation of these genes promotes and is critical during normal wound closure, and in the absence of such upregulation, as in diabetic rats, wound healing is severely impaired.

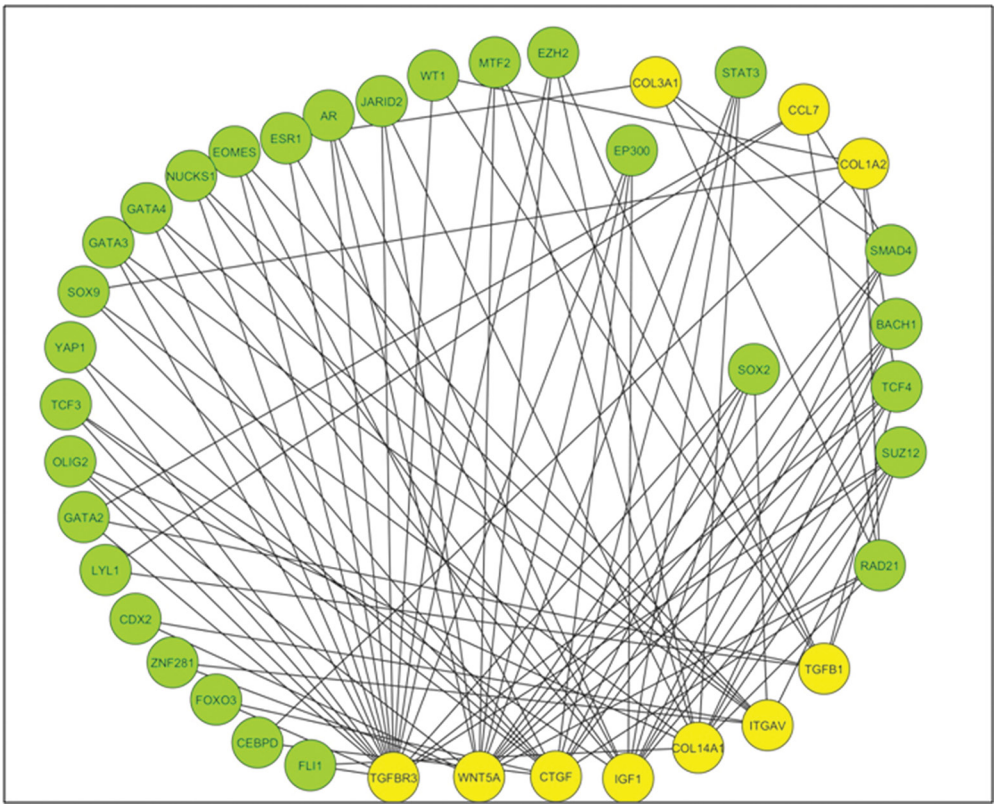

Figure 5. Network analysis among the predicted miRNA regulators and wound-healing genes. Regulators of the wound-healing genes altered in a diabetic rat wound (compared with normal rat wound, d 7 postwounding) were identified using the experimentally validated ChEA tool within ENRICHR. These were mapped onto the predicted targets of the 19 miRNAs altered (from Targetscan V. 7.0) in the same tissues. A total of 28 regulators were identified as potential miRNA targets, and networks of these prioritized regulators with the altered wound-healing genes were drawn using Cytoscape and a numerical value of 1.0. The green nodes are the miRNA-targeted regulators, and the yellow nodes are the altered wound-healing genes.

Interaction among the miRNA Predicted Targets and Altered Wound-Healing Genes

To further assess the crosstalk between the wound-healing genes and altered miRNAs, regulators to the altered wound-healing genes were extracted from ChEA within the ENRICHR tool. ENRICHR is an experimentally validated gene set enrichment tool that provides details of collective functions of gene lists. A list of 48 potential significant ($p < 0.05$) regulators of the 10 altered wound-healing genes were obtained. Looking for miRNA (rat) targeting these 48 regulators identified 28 regulators as being putative targets to the list of 19 altered miRNAs (http://www.targetscan.org/index.html). These 28 regulators were further examined for their functional enrichment by using the gene ontology online tool (http://geneontology.org/page/go-enrichment-analysis), and the PRC2 complex emerged as the most significantly enriched component (p = 5.83E-06 using Bonferroni correction for multiple testing). An interactive network between these 28 miRNA-targeted regulators and the wound-healing genes was drawn by using Cytoscape and is shown in Figure 5. The green nodes are miRNA predicted regulators, and the yellow nodes are the altered wound-healing specific genes (diabetic rat wound compared with normal rat wound, d 7 postwounding). Growth factors, namely *Ctgf*, *Igf1* and *Tgfβr3*, that are altered in the diabetic wound showed maximal interactions with the miRNA predicted regulators, suggesting that this axis might be regulated by miRNAs during the wound-healing process. Collagens (*Col1a2, Col3a1, Col14a1*) emerged as prominent nodes showing direct interactions with predicted miRNA-targeted regulators, suggesting a possible role of miRNAs in their regulation. Collagens are known to be critical during the process of wound healing. Wound strength depends on the balance between collagen synthesis and degradation. Impaired collagen synthesis inhibits wound closure, and collagen-based wound dressings are thought to be effective for delayed wound closure (34). *Wnt5a* and *Itgav*, components of the Wnt signaling and Integrin signaling pathways, respectively, also depicted multiple interactions with the miRNA predicted regulators. Integrins promote epithelial cell migration during normal wound-healing process and help in matrix formation. All these results suggest that the wound-healing genes that are altered in wounds of diabetic rats might be regulated by miRNAs via interaction with their predicted targets (regulators).

Inhibition of Dicer Prevents Keratinocyte Cell Migration

Because we observed downregulation of miRNAs and levels of Dicer were inhibited in the wounded tissues of diabetic rats (d 7 postwounding), we inhibited Dicer levels (using Dicer-specific siRNA) in HaCaT cells and assessed the effect on their migration patterns. HaCaT cells were transfected with the scramble or Dicer siRNA (10–100 nmol/L), and, after 24 h, cells were harvested to evaluate the levels of Dicer. As shown in Figures 6A and B, compared with scramble transfected cells, there was a dose-dependent decrease in Dicer levels in the presence of Dicer siRNA, both at the transcript and protein levels. Toward assessing the role of Dicer in wound closure, in scramble transfected cells, there was gradual migration of cells across the wound edge starting from as early as 6 h, evident by narrowing of the wound area. At 24 h, the wound area had closed and a monolayer of cells was seen. An almost similar pattern was observed in the cells transfected with 10 nmol/L Dicer siRNA. However, in cells transfected with 50 and 100 nmol/L of the Dicer siRNA, there was significant prevention of cell migration across the wound edge. At 6 and 12 h, the wound area remained comparable to that at 0 h, and even at 24 h, as opposed to the complete wound closure of the scramble transfected cells, an open wound area was still visible (Figures 6C, D). These data suggest that inhibition of Dicer prevents cell migration and delays wound closure.

DISCUSSION

In this study, we demonstrate downregulation of miRNAs during delayed wound closure in diabetic rats. Delay in wound healing is a frequently encountered event during diabetes. Diverse physiological factors that contribute to wound-healing delay include but are not limited to impaired growth factor production, collagen accumulation, keratinocyte migration and proliferation and angiogenic response. A major determining factor for such a delay is a prolonged inflammatory phase that increases the time required for wound closure during diabetes (35). This step impedes the start of the next phase of wound healing, thereby causing a delay in granulation and proliferation. Several therapeutic approaches have been reported that address these impairments, but there has not been much success in preventing delayed wound closure during diabetes.

The emergence of miRNAs as critical regulators of cellular processes has added another regulatory element in the wound-healing process. miRNAs have been identified to be critical in wound healing (24,36–38). Our results demonstrate an inhibited miRNA signature in the wounds of diabetic rats accompanied by decreased Dicer levels. Interestingly, our results also show that inhibiting the levels of Dicer in HaCaT cells prevent cell migration and wound closure.

Dicer, a member of the RNase III superfamily of bidentate nucleases mediates the processing of pre-miRNAs into mature miRNAs in the cytoplasm. Across all homologs of Dicer in diverse species, the enzyme contains a putative helicase domain and a Piwi Argonaute and Zwille (PAZ) domain, tandem nuclease domains and a double-stranded ribonucleic acid (dsRNA) binding domain (39). In addition to varied roles of Dicer, it has also been identified in the epidermal layer of skin and in the hair follicle. Dicer knockout (KO) mice show follicular epithelial progenitors evaginating into the epidermal surface instead of into the dermis (40,41). Notch and sonic hedgehog and epithelial

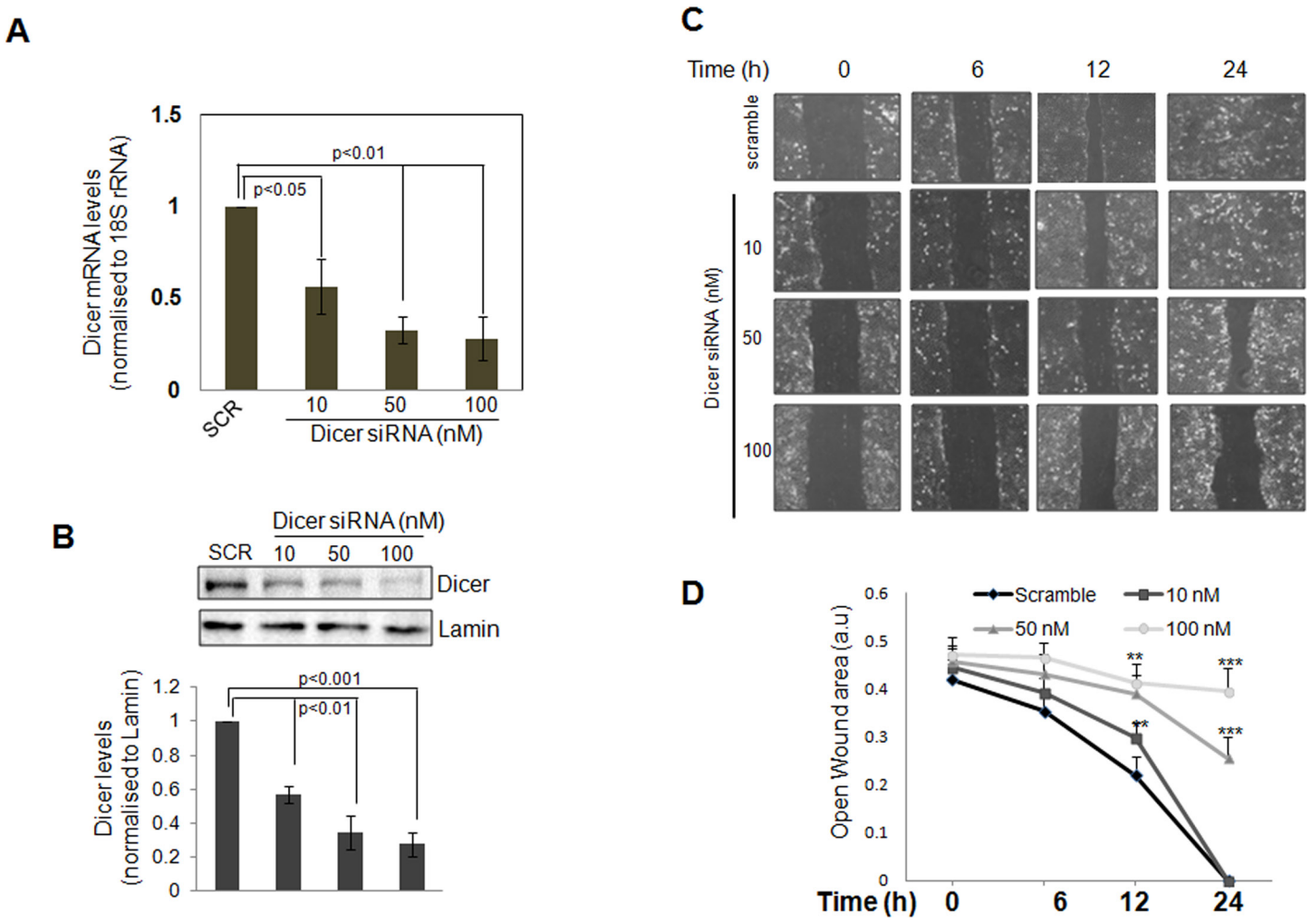

Figure 6. Inhibition of Dicer prevents HaCaT cell migration. (A) At ~40% confluence, HaCaT cells were transfected with the scramble (SCR) or Dicer siRNA (10–100 nmol/L) and, after 24 h, total RNA was isolated and the levels of Dicer mRNA was quantified by qRT-PCR, as described in Materials and Methods. 18S rRNA was used as the endogenous control. (B) Cells transfected as in (A) were lysed, and lysates (90 μg) were resolved on SDS-PAGE and subjected to Western blot analyses using anti-Dicer antibody. Lamin was taken as the loading control and densitometric analyses of the same are given in the panel below the Western blots. (C) HaCaT cells were transfected with either the scramble or Dicer siRNA and, after 24 h, a wound was made on the cell layer with a pipette tip. The movement of cells across the wound area at 6, 12 and 24 h was observed and photographed (10×). (D) The wound area at each time point in (C) was quantified. All experiments were in triplicate, and values are means ± SEM of three sets of independent observations. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the values in the presence of scramble at the same time points.

mesenchymal signaling are decreased by Dicer depletion, which results in deregulated skin physiology. These mice depict a marginal increase in apoptosis in the skin and start losing weight 1–2 d after birth (42,43). Oxidative insult can decrease Dicer levels as in observed in several pathological states (44,45). Although, in the context of endothelial cells, conditional Dicer inactivation reduces vascular endothelial growth factor (*Vegf*)-dependent angiogenesis and endothelial cell growth (46). These data suggest a critical role of Dicer in the skin. Our results demonstrate its involvement in keratinocyte migration, and decreased levels in a diabetic wound suggest that the levels of Dicer in the skin are presumably determining factors for impaired wound healing during diabetes. Interestingly, all the downregulated miRNAs in the diabetic rat wound (d 7 postwounding compared with normal rat wound) showed strong positive correlation with inhibited Dicer levels.

Our results show that compared with normal rat wounds (d 7 postwounding), several wound-healing specific genes are altered in the diabetic rat wound at this time point. *Col14a1, Col3a1 and Col1a2* family of collagen proteins were downregulated. Collagen accumulation and wound remodeling typically is the last

phase of the wound-healing process (47), and its inhibition, as seen in our study and also reported by others (34,48), is one of the main reason for the delay in wound closure. *Ctgf* and transforming growth factor β1 (*Tgfβ1*) were significantly upregulated. Both these factors are rapidly upregulated during injury and participate in initiating inflammatory events. However, in a diabetic wound, their chronic upregulation suggests the presence of excessive inflammation that is detrimental for wound repair. *Ctgf*, a cysteine-rich peptide of the CCN family (with CCN being an acronym for **C**onnetctive Tissue Growth Factor, **C**ysteine-rich protein 61, **N**ephroblastoma Overexpressed gene), has been shown to be involved in fibrogenesis during tissue healing, and Ctgf-deficient mice demonstrate impaired extracellular matrix production (49,50). Its levels are regulated by *Tgfβ*, and both these transcripts are coordinately expressed during wound repair (51). K5.Tgf$\beta 1^{wt}$ mice (transgenic mice overexpressing the wild-type *Tgfβ1* under the regulation of the K5 promoter) develop inflammatory skin disorders (52) and exhibit delayed wound healing (33). Wounds in these animals exhibit delayed reepithelialization and inflammatory cell infiltration compared with their nontransgenic counterparts. Persistent elevated levels of *Tgfβ1* promote recruitment of macrophages into the wound area that produce excessive proteases and inhibit reepithelialization and wound closure. On the other hand, *Tgfβ1* knockout mice display rapid epithelization and better wound-healing outcomes (53). While in a normal mouse, *Tgfβ* levels peak at d 3–4 postwounding and thereafter start decreasing, in a diabetic wound, *Tgfβ* levels start increasing from d 4, further increase on d 7 and remain elevated until d 13 (54). Overexpression of *Tgfβ1* in keratinocytes and hair follicles has been shown to promote inflammation, hyperplasia and angiogenesis (55,56). These data suggest that elevated levels of growth factors are possibly responsible for prolonged inflammation and delayed wound closure during diabetes. Integrin α V was significantly downregulated, suggesting that this might be responsible for the inhibited integration of membrane components and, therefore, of wound closure. In addition to angiogenesis and vasculogenesis, integrins are critical in cell–cell adhesion and adhesion to the extracellular matrix and thereby might contribute to impaired wound healing. *Ccl7* (a C-C motif chemokine 7) was increased by more than 2.5-fold, suggesting the presence of the inflammatory phase and the existence of inflammatory cells.

During the healing process in normal rats (normal rat skin wound [d 7 postwounding] compared with unwounded normal rat skin), eight genes (*Rac1*, *Col14a1*, *Igf1*, *Plat*, *Mapk1*, *Ctnnb1*, *Itga4* and *Rhoa*) were upregulated and showed no significant alteration during the delayed healing process in diabetic rat wounds (diabetic rat skin wound [d 7] compared with unwounded diabetic rat skin), suggesting that these genes are possibly critical in promoting normal wound closure. By not being altered during delayed wound closure in diabetic rats, they are presumably preventing the timely closure of wounds. *Igf1* is induced in wounds, both in animals (57,58) and humans (59,60), but is missing in chronic wounds (61). Proliferation and migration of keratinocytes and fibroblasts is promoted by *Igf1 in vitro* (62,63) and, interestingly, its levels are reduced in cells isolated from diabetic foot ulcers (64) and in diabetic mouse wounds (65). *Igf1* treatment or overexpression has been shown to promote wound healing in diabetic animals (66,67). *Plat* (tissue-type plasminogen activator) was also significantly upregulated during healing in normal rats, but was notably missing during impaired healing in diabetic rats. It is a secreted serine protease that converts the proenzyme plasminogen to plasmin and plays a critical role in cell migration and tissue remodeling. Keratinocytes use plasmin activated by plasminogen activators for paving their way through the matrix during wound healing (68). Plasminogen-deficient mice depict a delay in wound healing, as evidenced by decreased migration of keratinocytes from the wound edges and their decreased ability to proteolytically dissect their way through the matrix (69,70). Another upregulated gene, *Ctnnb1*, also known as β-catenin, belongs to the adherens junction proteins that mediate adhesion between cells and is important in cell communication, growth, embryogenesis and wound healing (71). Its levels are elevated in mesenchymal cells during the proliferative phase of wound repair (72), and it is thought that *Ctnnb1* regulates the growth of dermal fibroblasts (73,74). Macrophage-specific deletion of β-catenin in mice shows defects in migration and adhesion to fibroblasts and consequently inefficient skin wound healing. Macrophages overexpressing β-catenin rescued impaired wound-healing in irradiated mice (75). Rac1, another protein upregulated in normal rats during wound healing, participates in the coordinated and orchestrated process of wound healing. Along with RhoA, another member of the small GTP-binding family of Rho-GTPases, Rac1 is required for the reorganization of the cytoskeleton for efficient wound closure (76).

Also, several regulators of the altered wound healing genes were identified to be predicted targets of the altered miRNAs. This result suggests that miRNAs that are deregulated in the wound during diabetes might be indirectly responsible for the differential status of wound-healing genes. These interactions are an area worthy of investigation.

CONCLUSION

Our results provide evidence for an altered miRNA and wound-healing genes' status that is associated with the prolonged inflammatory phase in the wounds of diabetic rats. The downregulated miRNA signature is presumably due to decreased levels of Dicer and, to the best of our knowledge, this is a novel finding of an inhibited status of Dicer in a wound during diabetes and of its critical involvement in

delaying wound closure. A recent report demonstrates compromised wound closure in keratinocyte-specific Dicer-ablated mice, suggesting the significance of Dicer in keratinocytes during restoration of skin function postwounding. Absence of Dicer in these animals and the consequent downregulation of miRNAs were responsible for desilencing $p21^{Waf1/Cip1}$ expression, and this disrupted the skin barrier function. The authors suggest that $p21^{Waf1/Cip1}$ might be a potential drug target to improve barrier function of the skin during Dicer loss (44). Identification of decreased Dicer levels during impaired wound healing in diabetes in our study, therefore, offers a valuable tool to be addressed such that the delay in the wound-healing process during diabetes might be targeted.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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