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# **The Role of MicroRNAs in Impaired Diabetic Wound Healing**

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Additional information is available at the end of the chapter

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## **Abstract**

Diabetes mellitus is a worldwide pandemic, affecting 29 million Americans, resulting in substantial morbidity and mortality, and accounting for an annual healthcare expenditure exceeding \$176 billion in the US alone. This burden of disease is the result of a progressive disease associated with numerous complications and the development of chronic wounds, which remain the leading cause of hospital admissions and nontraumatic lower extremity amputations in diabetic patients. Despite clinical strategies aimed at prevention and early detection, patients with diabetes continue to remain at risk of developing chronic diabetic wounds due to poor patient compliance and progression of the diabetic phenotype. Development of the diabetic phenotype and wound healing impairment is associated with dysregulation of microRNAs that regulate inflammation, extracellular matrix composition, and angiogenesis; here we present evidence from the studies that demonstrate correction of microRNA dysregulation expedites wound healing and reverses the diabetic skin phenotype.

**Keywords:** microRNA, diabetic wound healing, inflammation, angiogenesis, extracellular matrix

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## **1. Introduction**

Diabetes mellitus is a worldwide pandemic, affecting 29 million Americans and over 171 million peoples worldwide, resulting in substantial morbidity and mortality, and accounting for an annual healthcare expenditure exceeding \$176 billion in the US alone (Data from the 2014 National diabetes fact sheet; available at <http://www.cdc.gov/diabetes/pdfs/data/2014-report->

estimates-of-diabetes-and-its-burden-in-the-united-states.pdf). This burden of disease is the result of a progressive disease process associated with numerous complications, such as retinopathy, neuropathy, and nephropathy, as well as the development of chronic wounds. Chronic wounds remain the leading cause of hospital admissions and nontraumatic lower extremity amputations in patients with diabetes, with nearly 80% of all amputations performed in patients with diabetes preceded by a diabetic wound [1]. Risk factors for the development of diabetic wounds include the presence of the “pathogenic triad” of neuropathy, ischemia, and trauma [2–4]. In addition, foot deformities, lower extremity edema, and use of inappropriate footwear also contribute to the development of diabetic wounds [2–4].

Despite clinical strategies aimed at prevention and early detection of diabetic neuropathy and diabetic wounds, patients with diabetes continue to have a 12–25% lifetime risk of developing a chronic diabetic wound due to poor patient compliance and progression of the diabetic phenotype [5, 6]. Projections indicate that by 2025 diabetes will affect over 300 million people worldwide, with a rising proportion of the burden borne by patients in developing countries [7, 8]. Given this anticipated rise in the burden of disease attributable to diabetes, and the potential concomitant rise in development of chronic wounds, the impetus for developing more effective wound care strategies cannot be understated.

After injury, patients with diabetes suffer from an impaired wound healing response; the standard wound healing response, in which the tissue passes through consecutive, but overlapping phases of coagulation, inflammation, proliferation, and remodeling, is disrupted [2]. Notably, patients with diabetes demonstrate a phenotype characterized by decreased angiogenesis, impaired leukocyte migration, decreased growth factor production, sustained inflammation, impaired fibroblast function, and imbalance of extracellular matrix deposition and remodeling, and delayed wound healing [9]. A longer duration of diabetes has been associated with a greater risk of impaired wound healing; consistent with this observation, duration of diabetes has been correlated with more profound diabetic neuropathy, increasingly compromised biomechanical properties of diabetic skin, and an increasingly delayed rate of endothelial progenitor cell proliferation. While the cytokines, chemokines, and cellular components of the wound healing response have been extensively studied, recent attention has focused upon the role that microRNAs (miRNAs) play in the impaired healing of diabetic wounds [3].

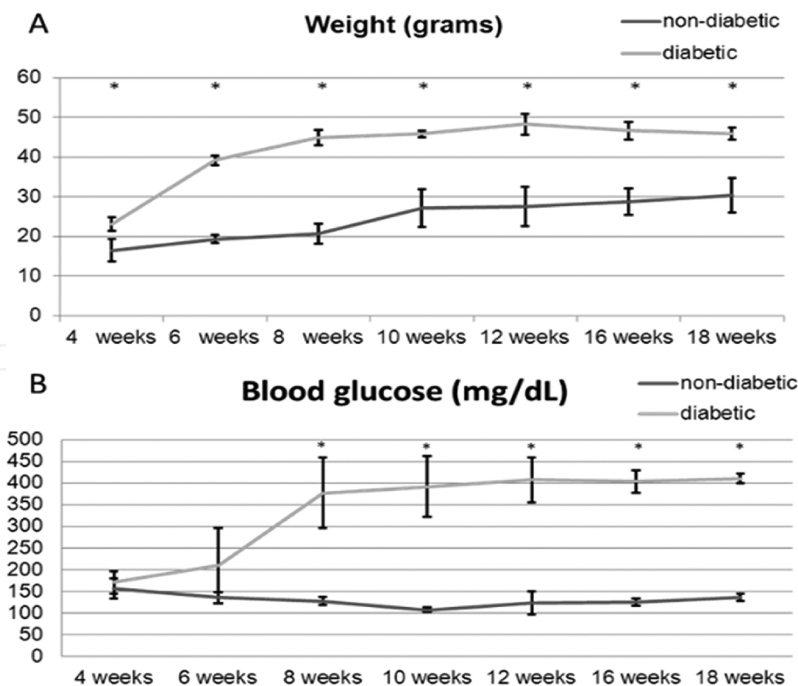
MiRNAs are a class of small, noncoding RNA molecules, 20–22 nucleotides long, that regulate gene expression at the posttranscriptional level [10]. Complementary binding of miRNAs to the 3′-untranslated region (UTR) of their target messenger RNA (mRNA) results in posttranscriptional repression and/or mRNA degradation, thereby regulating the expression of downstream targets [11]. MiRNA are thought to regulate over one-third of all physiologic processes, including regulation of cell cycle progression, apoptosis, cytokine transcription, cellular metabolic function, signal transduction, proliferation, and determination of cell fate [12, 13]. MicroRNAs are being investigated for their role as biomarkers, diagnostic tools, and therapies in a variety of disease states, including diabetes and numerous types of cancer [14, 15]. Key to the development of the diabetic phenotype is the dysregulation of the expression of miRNA that regulate inflammation, extracellular matrix composition, and angiogenesis.

Here we discuss data that demonstrate correction of microRNA dysregulation expedites wound healing and reverses the diabetic phenotype in skin [16–18].

## 2. Methods

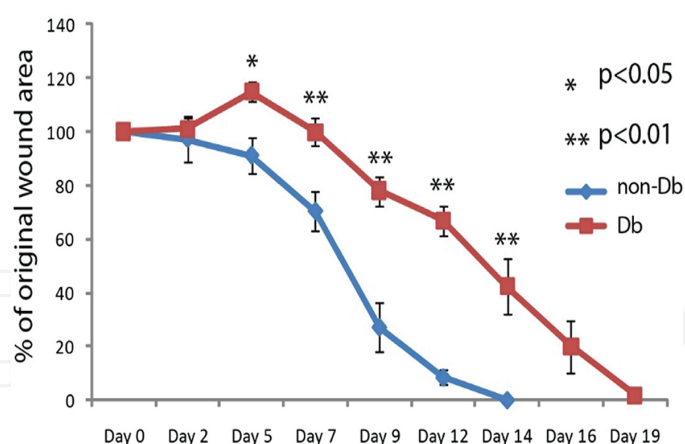
### 2.1. Murine model of diabetic wound healing

We utilized a murine model of diabetes, using genetically diabetic female mice homozygous for the leptin receptor mutation (db/db, C57BKS.Cg-m-/-*Leprdb/J*) and age-matched, nondiabetic, heterozygous controls (db/-), obtained from the Jackson Laboratory (Bar Harbor, ME). Leptin is a centrally acting hormone that modulates both satiety and body fat content [19, 20]. Mice homozygous for the leptin receptor mutation develop hyperphagia, obesity, hyperglycemia, insulin resistance, and hyperlipidemia, leading to their extensive use as a murine model of diabetes [19, 20]. Between 4 and 6 weeks of age, the diabetic mice are significantly more obese and hyperglycemic than their nondiabetic counterparts (**Figure 1**), and by 10–14 weeks of age, diabetic mice weigh greater than 45 g, with serum glucose levels in excess of 400 mg/dL, whereas nondiabetic mice weighed less than 25 g, with serum blood glucose levels less than 250 mg/dL [21]. Most noteworthy for our analysis, diabetic mice also demonstrate significantly delayed wound healing when compared with nondiabetic mice, requiring up to 5 days longer to close cutaneous wounds (**Figure 2**) [21, 22].



**Figure 1. Weight (A) and serum blood glucose (B) of diabetic and nondiabetic mice.** (A) Weight of diabetic (C57BKS.Cg-m-/-*Leprdb/J*) and nondiabetic (C57BKS.Cg-m-/+*Leprdb/J*) mice at different ages. (B) Serum blood glucose levels in diabetic and nondiabetic mice at different ages. Asterisk (\*) indicates  $p < 0.05$  for any given time point (Image reproduced from Zgheib *et al.* [21] with permission of the authors.)





**Figure 2. Wound closure in diabetic and nondiabetic mice.** The percent (%) of original wound area remaining at various time points during the wound healing process in diabetic and nondiabetic mice. Asterisk (\*) indicates  $p < 0.05$  and (\*\*) indicates  $p < 0.001$ .

To obtain a baseline, unwounded skin from the dorsum of diabetic and nondiabetic mice was harvested, and the cranial-caudal orientation was preserved for the purposes of biomechanical testing. Diabetic and nondiabetic mice between 10 and 14 weeks of age had full thickness, excisional dermal wounds created using an 8 mm dermal punch biopsy (Miltex, Inc., York, PA) through the panniculus carnosus, as previously described [17, 21]. Following the initial wounding, mice were treated with either phosphate buffered saline (PBS),  $10^6$  mesenchymal stem cells (MSCs), and  $1 \times 10^6$  plaque forming units (PFU) of either an empty lentiviral vector or a lentiviral construct containing a mutated stromal cell derived factor (SDF-1 $\alpha$ ) transgene, or 10  $\mu$ L of 100 ng/mL of recombinant human SDF-1 $\alpha$  protein. The wounded skin was harvested at 1, 3, 7, 14, and 21 days after initial wounding and animals were euthanized by inhalation of carbon dioxide followed by cervical dislocation.

## 2.2. Isolation of MSCs

MSCs were isolated from the femurs and tibia of adult C57BL/6TbN (act-GFP) Osby01 transgenic (GFP) mice, as described previously [17]. Mononuclear cells were then separated by density gradient separation using Ficoll before being suspended at a density of  $2.5 \times 10^4$  cells/ $\mu$ L in PBS [23].

## 2.3. Human skin analysis

Human skin samples were obtained via the National Disease Research Interchange (NDRI). Human skin samples measuring  $5 \times 5$  cm were obtained from the anterior portion of the lower extremities within 8 h postmortem. The samples were obtained from patients who were between 45 and 75 years old, without significant comorbidity, malignancy, or a history of radiation or chemotherapy. While it was known whether or not the patients carried an existing diagnosis of diabetes, as well as what medications the individual was currently taking, data regarding the duration of diabetes, degree of blood glucose control, or intensity of sun

exposure was not available. Following receipt of the sample, subcutaneous tissues were sharply excised from the dermis and the skin samples intended for biomechanical analysis were immediately flash frozen in liquid nitrogen.

#### 2.4. Culture of dermal fibroblasts

Human skin samples and murine skin samples were processed in order to enable the culture of dermal fibroblasts for further *in vitro* analysis. Skin samples were washed in 70% ethanol for 2 min, followed by 3 washes in PBS. Subcutaneous tissues were sharply excised from overlying dermis, and the remaining dermis was minced before being digested in 2 mL of 1000 u/mL Collagenase for 1 h at 37°C. The sample was then centrifuged at 1000 rpm for 5 min, the supernatant was removed, and the sample was then digested in 5 mL of dispase (1.9 u/mL) for 30 min at 37°C. The sample was again centrifuged at 1000 rpm for 5 min, the supernatant was removed, and the remaining pieces of dermis were mixed with 10 mL of DMEM with 10% fetal bovine serum (FBS, Life Technologies, Carlsbad, CA) and 1% antibiotic-antimycotic (Life Technologies, Carlsbad, CA) and plated in a 100 mm tissue culture plate (Corning Incorporated, Corning, NY). When fibroblasts were observed to proliferate and adhere to the tissue culture plate independent of the pieces of dermis, the dermis was removed, the plate was washed twice with 10 mL PBS, and the culture media was replaced. Cells between passage 1 and 4 were used for these experiments.

#### 2.5. Lentiviral construction

The SDF-1 $\alpha$  mutant that was utilized binds the CXCR4 receptor without activating it [24, 25]. According to the manufacturer's instruction, a complementary DNA (cDNA) library was prepared from mouse tissues using TRIzol and Superscript (Invitrogen, Carlsbad, CA). After sequence analysis confirmed the SDF1 $\alpha$  cDNA and the SDF1 $\alpha$  inhibitor (SDF $\alpha$ i), the CS-CG HIV-1 transfer plasmid was used to generate a self-inactivating lentiviral vector designed to convey expression of either the green fluorescent protein reporter gene (GFP, Clontech Laboratories, Mountain View, CA) or the mutant SDF1 $\alpha$ i with the GFP reporter gene under the control of a cytomegalovirus promoter [25–27]. The ability of the plasmid to effectively transfect cells was tested on murine dermal fibroblasts *in vitro* prior to *in vivo* use.

#### 2.6. Biomechanical testing

In order to assess the biomechanical properties of murine and human diabetic and nondiabetic skin, all samples immediately underwent biomechanical testing after harvesting. Biomechanical testing was performed on the diabetic and nondiabetic murine samples at baseline, 4 weeks, and 7 weeks after wounding. Prior to testing, the subcutaneous tissues were removed, and a uniform dumbbell-shaped testing unit was stamped out from the sampled skin [28, 29]. The cranial–caudal orientation of each sample was maintained, and the healed wound, if present, was centrally located within the testing unit. Two lines of Verhoeff stain were placed on either end of the sample, thereby identifying the length of the testing area. The mean cross-sectional area of each sample was then measured using a custom laser band [30]. Each sample

underwent testing to calculate the modulus of elasticity and the maximum stress until failure, as previously described [29].

## 2.7. Immunohistochemistry

Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol. GFP was detected with application of rabbit anti-GFP primary antiserum (1:100; Invitrogen, Carlsbad, CA) followed by biotinylated antirabbit secondary antibody (1:200), and the slides were developed using avidin–biotin complex (Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin. For double-immunofluorescent staining, sections were blocked with 1% sodium borohydrate (Sigma–Aldrich, St. Louis, MO) in PBS. Platelet endothelial cell adhesion molecule-1 (CD31) was used as an endothelial marker. CD31 was detected by using rat anti-mouse CD31 antibody (1:20 dilution; Invitrogen, Carlsbad, CA and AbCam, Cambridge, MA) followed by biotinylated rabbit anti-rat secondary antibody and detection with the alkaline phosphatase detection system (Vector Laboratories, Burlingame, CA). GFP was detected by using the same rabbit anti-GFP primary antiserum followed by Alexa-Fluor-488-conjugated F(ab')<sub>2</sub> secondary antibodies (1:100; Invitrogen). Slides were counterstained with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA).

Sections were stained for cluster of differentiation (CD) 45, which was used as a cellular marker for inflammatory cells, and antigen retrieval was performed using 13 Antigen Retrieval Citra (BioGenex HK086-9k, Fremont, CA) in a decloaking chamber using the factory default settings (Biocare Medical, Concord, CA). Slides were rinsed three times in 0.1% PBST (0.1% Triton100 in PBS), blocked in 20% goat serum in 0.4% PBST for 60 min at room temperature, and incubated overnight at room temperature with primary antibodies against CD 45 (rabbit polyclonal ab10558, 1:100; Abcam, Cambridge, MA) in 5% goat serum and 0.1% PBST. The following day, slides were washed three times in 0.1% PBST and incubated with the appropriate anti-rabbit biotinylated secondary antibodies for 1 h at room temperature. Slides were washed three times in 0.1% PBST and mounted in Vectashield (Vector Laboratories, Burlingame, CA). The slides were then incubated with avidin–biotin–peroxidase complex (Vector Laboratory) and developed, as described by the manufacturer. The mean number of either CD45 or CD31 positive cells per high power field (hpf) was calculated as the average of 5 hpf per slide.

## 2.8. Enzyme-linked immunosorbent assay and Western Blot

Skin samples had their subcutaneous tissues removed before being flash frozen in liquid nitrogen immediately after harvest. Collagen content was quantified by Western Blot. Skin samples were cut into 1-mm pieces and homogenized in a 0.5 M acetic acid solution containing 1× protease inhibitor cocktail and 5 mmol/L EDTA, in order to solubilize total collagen. The solution was then centrifuged for 4 h at 4°C at  $16.1 \times 10^3$  G, the lipid layer was aspirated, and this process was repeated. Bicinchoninic acid protein assay was used to quantify the total protein concentration of the supernatant, and Western blot was performed using standard techniques, and final protein concentrations were standardized to 1 µg/2 µL using a sample buffer before being boiled at 95°C for 5 min. Tris–acetate gels (3–8%) were run at 150 V for 1 h,

transferred to 30 V overnight at 4°C, rinsed with Tris-buffered saline, and were subsequently blocked with 5% milk in Tris-buffered saline with 0.1% Tween for 1 h. Collagen I antibody (Abcam Inc., Cambridge, MA) was diluted to 1:2000 (collagen III to 1:1000) and blots were incubated for 1 h at room temperature, washed with Tris-buffered saline with 0.1% Tween, and incubated with secondary antibody (anti-rabbit IgG horseradish peroxidase; GE Healthcare, Marlborough, MA) at 1:10,000 for 1 h at room temperature. Western blots were washed with Tris-buffered saline with 0.1% Tween and then Tris-buffered saline. The blots were then developed using a standard chemiluminescence solution (ECL solution A and B; GE Healthcare, Marlborough, MA) and incubated for 1 min.

## **2.9. Real-time polymerase chain reaction**

Total RNA was extracted and purified from skin samples after homogenization in TRIzol (Invitrogen and Life Technologies, Carlsbad, CA), following the manufacturer's instructions. RNA was converted into cDNA using the SuperScript First-Strand Synthesis System (Invitrogen and Life Technologies, Carlsbad, CA). Random primers were used for the reverse transcription reaction and real-time quantitative polymerase chain reaction (PCR) was performed with either the CFX96 real-time PCR thermal cycler (Bio-Rad, Hercules, CA) or the ABI 7900 realtime PCR thermal cycler (Applied Biosystems, Foster City, CA). These samples were amplified in triplicate using primers for NFkB, IRAK1, TRAF6, IL-6, MIP-2 (IL-8), col1a2, col3a1, VEGF, BCL-2, HIF-1 $\alpha$ , miR-146a, miR-15b, and miR-29a (TaqMan gene expression assay, Applied Biosystems, Foster City, CA). Internal normalization was achieved by using the 18 s housekeeping gene as an internal control for mRNA and the U6 housekeeping gene as an internal control for miRNA.

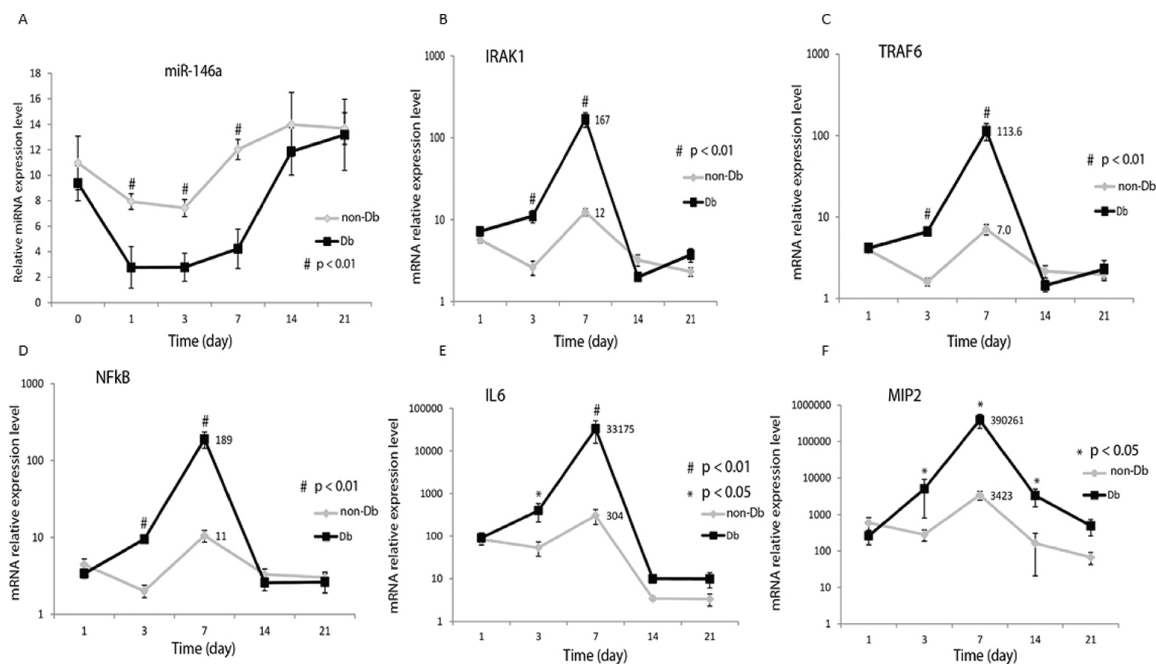
## **3. Impaired wound healing observed in diabetes is associated with impaired microRNA expression**

### **3.1. Sustained inflammation: the role of mir-146a**

While inflammation plays an integral role in normal wound healing, the presence of pathologically sustained inflammation is a chief component of the dysregulated wound healing observed in patients with diabetes [15, 31]. The inflammatory phase of wound healing is characterized by increased infiltration of inflammatory cells (neutrophils and macrophages) and release of inflammatory mediators, such as cytokines [32]. Both the cellular and cytokine response to injury are mediated by microRNA. Specifically, miR-146a has been identified as a key regulator of the nuclear factor kappa-B (NFkB) pathway, which is known to regulate numerous inflammatory processes, as well as the transcription of several inflammatory cytokines [33, 34]. Following activation of the NFkB pathway by toll-like receptors (TLRs), NFkB expression is positively regulated by interleukin-1 receptor-associated kinase 1 (IRAK1) and TNF receptor associated factor 6 (TRAF6) [35]. Increased activity of IRAK1 and TRAF6 result in increased NFkB activity, which then upregulates the expression of genes coding for

the key proinflammatory cytokines interleukin-6 (IL-6) and IL-8 [17]. However, NFkB activity can also induce the expression of miR-146a, which inhibits IRAK1 and TRAF6, thereby acting as a brake on the NFkB dependent innate immune response [36].

Analysis of skin samples obtained from wounded diabetic and nondiabetic mice demonstrates significant downregulation of the anti-inflammatory miR-146a in diabetic mice during the course of wound healing (**Figure 3**) [17]. In addition to down regulation of miR-146a, wounded diabetic skin demonstrates significantly elevated expression of mRNA coding for IRAK1, TRAF6, NFkB, and the proinflammatory cytokines IL-6 and IL-8 (with MIP2 being the murine equivalent of IL-8) (**Figure 3**) [17].



**Figure 3. Quantification of miRNA-146a and components of the NFkB pathway in diabetic and nondiabetic wounds.** Real-time PCR quantification of miR-146a (A), IRAK1 (B), TRAF6 (C), NFkB (D), and the downstream end products IL-6 (E) and MIP2 (F) (the murine equivalent of IL-8) days 0–21 after wounding in diabetic and nondiabetic mice. Results are presented as a mean + SEM for each cohort at each time point. Asterisk (\*) indicates  $p < 0.05$  and # indicates  $p < 0.01$ . (Image reproduced from Xu *et al.* [17] with permission of the authors.)

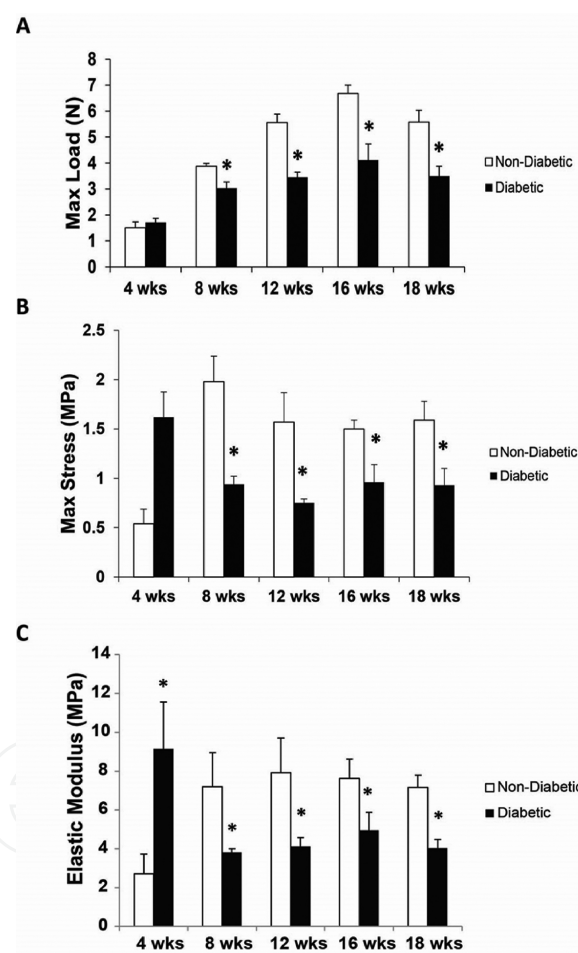
### 3.2. Impaired biomechanical properties and deposition of extracellular matrix: the role of miR-29a

In addition to dysregulation of the maturation phase of wound healing, diabetic skin has been shown to be biomechanically impaired at baseline, with decreased maximum load, maximum stress prior to failure, and decreased elasticity, as seen in **Figure 4** [21, 29]. It is thought this baseline impairment is one of the many factors that place even intact diabetic skin at a higher risk of injury than nondiabetic skin, with continued dysregulation of extracellular matrix remodeling contributing to impaired healing after injury [15, 29]. In addition, the balance of matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs)



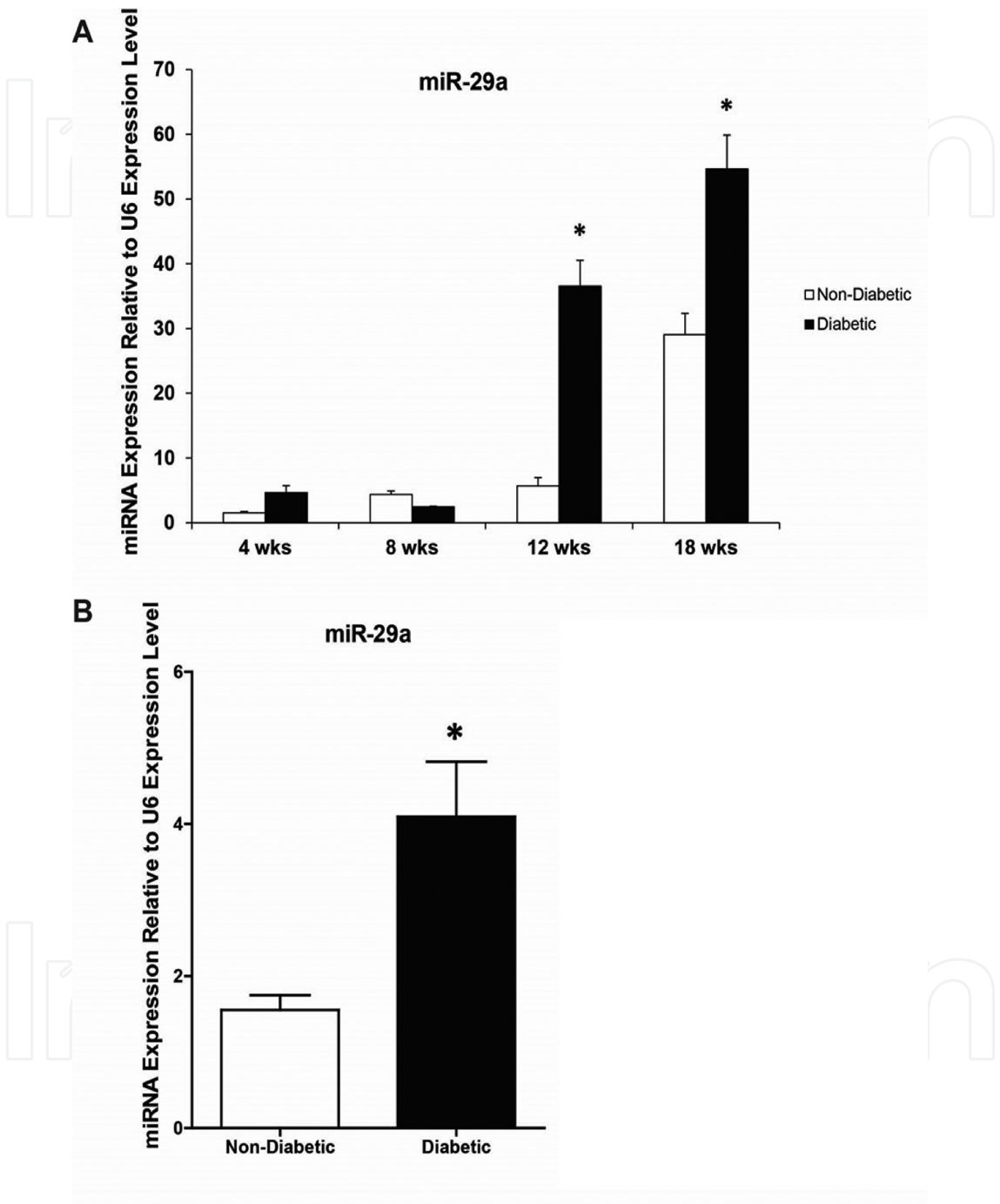
in diabetic wounds is weighted toward increased breakdown of extracellular matrix components, contributing to poor wound healing [2, 31, 37].

MiR-29a contributes to this impaired extracellular matrix remodeling by posttranscriptional regulation of collagen content, leading to an inverse relationship between miR-29a levels and collagen content [38, 39]. **Figure 5** details the significant upregulation of miR-29a gene expression that has been detected in both diabetic murine (**Figure 5A**) and diabetic human skin (**Figure 5B**). This miR-29a dysregulation corresponded to elevated gene expression of collagen 1 $\alpha$ 2 (col1 $\alpha$ 2) and collagen 3 $\alpha$ 1 (col3 $\alpha$ 1) in murine diabetic skin when compared with nondiabetic skin (**Figure 6**); however, Western blot confirmed decreased levels of both col1 $\alpha$ 2 and col3 $\alpha$ 1 protein in diabetic murine skin, versus nondiabetic murine skin (**Figure 6**). During the maturation phase of wound healing, the extracellular matrix undergoes remodeling



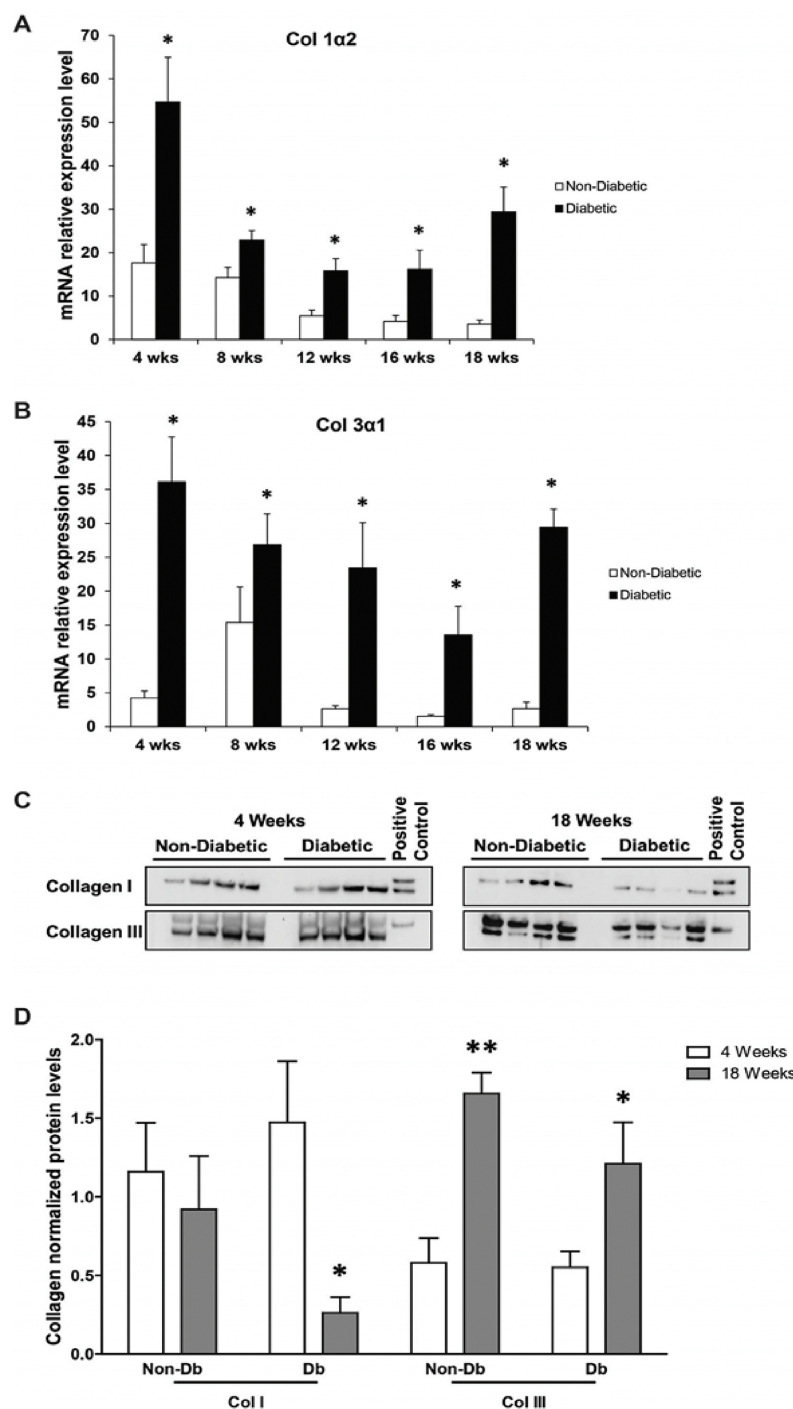
**Figure 4. Baseline biomechanical properties of diabetic and non-diabetic skin.** (A) The maximum load sustained (N) prior to failure in diabetic versus nondiabetic skin samples over 4–18 weeks of age. (B) The maximum stress to failure (MPa) in diabetic versus nondiabetic skin samples over 4–18 weeks of age. (C): The elastic modulus (MPa) measured in diabetic versus non-diabetic skin samples over 4–18 weeks of age. Data is presented as a mean + standard error of the mean (SEM) for each cohort. Student's *t*-test was used to compare nondiabetic skin vs. diabetic skin at each time point. Asterisk (\*) indicates  $p < 0.05$ . Abbreviation: Max = Maximum. (Image reproduced from Zgheib *et al.* [21] with permission of the authors.)

characterized by type III collagen being replaced by type I collagen, and this process is thought to be impaired in diabetic skin [15].



**Figure 5. MiRNA-29a gene expression in diabetic and nondiabetic murine (A) and human (B) skin. (A)** Real-time quantitative PCR analysis of miRNA-29a levels in murine diabetic and nondiabetic skin at different age-points. **(B)** Real-time quantitative PCR analysis of miRNA-29a levels in human diabetic and nondiabetic skin. MiR-29a gene expression was calculated after normalizing with U6. Results are presented as a mean + SEM for each cohort. Student's *t*-test was used to compare nondiabetic skin to diabetic skin at each time point. Asterisk (\*) indicates  $p < 0.05$ . (Image reproduced from Zgheib *et al.* [21] with permission of the authors.)



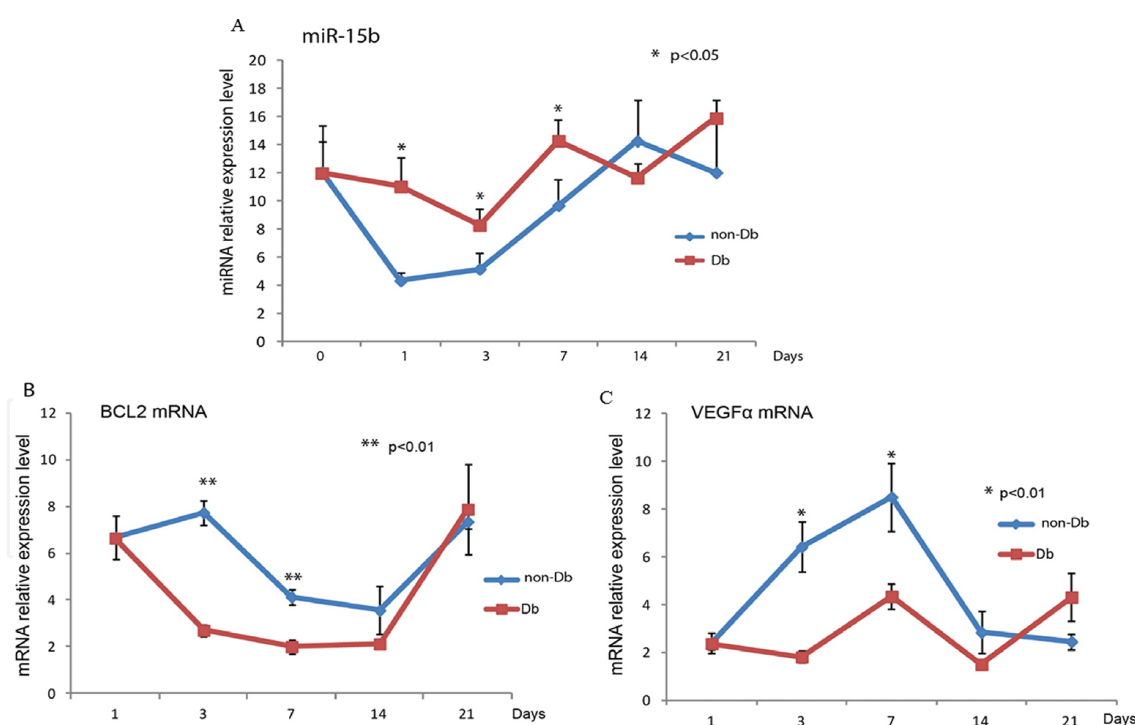


**Figure 6. Collagen gene and protein expression in diabetic and non-diabetic murine skin.** (A) Relative gene expression for collagen 1α2 in skin samples from diabetic (n = 5) and non-diabetic (n = 5) mice from 4 to 18 weeks of age. (B) Relative gene expression for collagen 3α1 in skin samples from diabetic (n = 5) and non-diabetic (n = 5) mice from 4 to 18 weeks of age. (C) Collagen I and III protein levels (upper band; black arrows) as demonstrated by western blots, obtained from skin samples from age-matched, non-diabetic and diabetic mice at 4 and 18 weeks of age. (D) Collagen I and III protein levels as quantified by western blot. These findings are representative of five independent experiments. Data is presented as a mean + SEM for each cohort. Student's *t*-test was used to compare non-diabetic skin to diabetic skin at each time point. Asterisk (\*) indicates *p* < 0.05; \*\* indicates *p* < 0.001. (Image reproduced from Zgheib *et al.* [21] with permission of the authors.)

### 3.3. Decreased angiogenesis: the role of miR-15b

Successful angiogenesis requires coordinated extracellular matrix production in order to provide an adequate architecture for formation of new blood vessels [31]. However, angiogenesis within a wound bed is further regulated by numerous angiogenic factors, with vascular endothelial growth factor (VEGF) considered one of the most prominent [40]. Following injury, hypoxia in the wound bed leads to increased expression of hypoxia inducible factor-1 (HIF-1), a transcription factor that increases the expression of numerous proangiogenic proteins, including VEGF [31, 41]. In turn, VEGF attracts endothelial cells to the site of injury, in addition to inducing proliferation and angiogenesis via upregulation of proteins, such as the anti-apoptotic B-cell lymphoma-2 (BCL-2) [16, 40]. BCL-2 is also thought to improve wound healing by stabilizing the alpha subunit of HIF-1 (HIF-1 $\alpha$ ), mediated by heat shock protein 90 (HSP90), thereby increasing HIF-1 mediated VEGF expression [16]. When compared to nondiabetic murine wounds, diabetic murine wounds have been detailed to have decreased levels of HIF-1 $\alpha$  activity, VEGF gene expression, and BCL-2 gene expression, as well as significantly decreased number of cells that stain for the presence of platelet endothelial cell adhesion molecule 31 (CD31), a marker of endothelial cells [42].

MiR-15b negatively regulates angiogenesis by decreasing VEGF expression; this decrease in VEGF expression is associated with decreased cell migration and vascular tubule formation



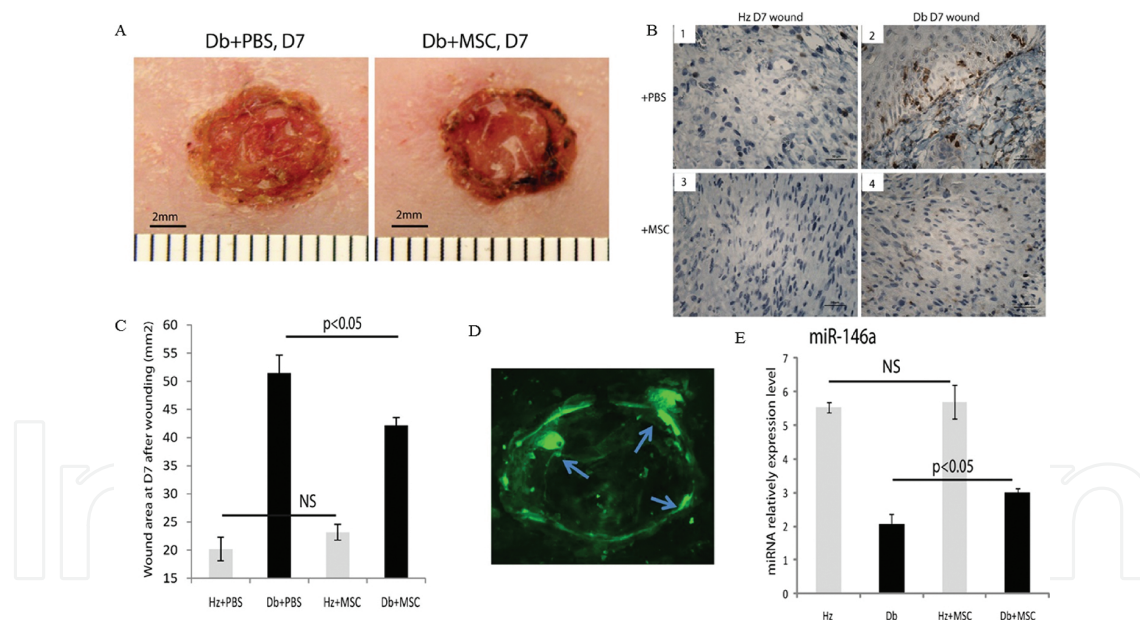
**Figure 7. Quantification of miRNA-15b, BCL2, and VEGF $\alpha$  gene expression in diabetic and non-diabetic wounds.** Quantification by real-time PCR of miRNA-15b (miR-15b) (A), BCL2 (B), and VEGF $\alpha$  (C) gene expression in murine diabetic wounds versus murine non-diabetic wounds, 3–7 days after wounding. Results are presented as a mean + SEM for each cohort at each time point. Asterisk (\*) indicates  $p < 0.05$  and \*\* indicates  $p < 0.001$ . (Image reproduced from Xu *et al.* [16] with permission of the authors.)

*in vitro* [43]. In nondiabetic humans and mice, the hypoxic conditions following wounding decrease the expression of miR-15b, leading to increased levels of HIF-1 $\alpha$ , VEGF, and BCL-2. However, in our murine model of diabetic wound healing, miR-15b expression was significantly upregulated in diabetic mice compared to nondiabetic mice 1, 3, and 7 days after wounding (Figure 7). Furthermore, the upregulation in miR-15b expression observed in diabetic mice was associated with a significant downregulation in VEGF and BCL-2 gene expression 3 and 7 days after wounding (Figure 7).

## 4. Therapeutic targets

### 4.1. The impact of cellular therapies on diabetic wound healing

In the setting of the tremendous clinical and fiscal burden of chronic diabetic wounds, efforts to develop effective wound care strategies are ongoing. The dysregulation of wound healing in patients with diabetes occurs at every stage of healing—whether it be the inflammatory phase, the proliferative phase, or the remodeling phase. Given this widespread dysregulation, therapies directed at individual targets of the wound healing response are unlikely to be completely successful in addressing the diabetic wound healing impairment. As such,

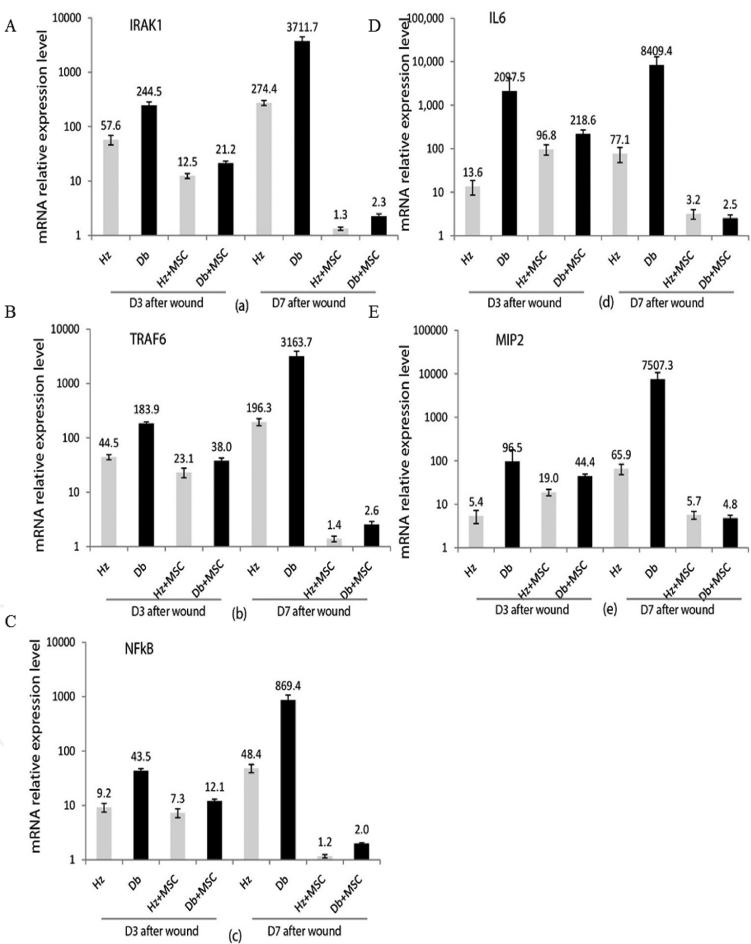


**Figure 8. Treatment of diabetic wounds with MSCs expedites wound closure and upregulates miRNA-146a, 7 days after wounding.** (A) Diabetic wounds treated with PBS (left) or MSCs (right). (B) CD45 immunostaining of diabetic (Db) and nondiabetic (Hz) wounds, following treatment with either PBS or MSCs. (C) Quantitative assessment of diabetic (Db) and nondiabetic (Hz) wound closure, following treatment with either PBS or MSCs. (D) Fluorescent image demonstrating GFP positive cells (MSCs), confirming the persistence of MSCs after injection. Blue arrows indicate injection sites. (E) Real-time PCR confirms the upregulation of miRNA-146a gene expression in Db treated with MSCs, compared with Db wounds treated with PBS. Results are presented as a mean + SEM for each cohort at each time point. Asterisk (\*) indicates  $p < 0.05$  and \*\* indicates  $p < 0.001$ . (Image reproduced from Xu *et al.* [17] with permission of the authors.)

attention has been drawn to the use of cell-based therapies for the treatment of chronic diabetic wounds, with the hopes that multipotent cell therapy will address impaired diabetic wound healing at multiple levels of dysregulation [41]. Specifically, MSCs have been a focus due to their capacity for self-renewal, multipotency, and their ease of retrieval from autologous bone marrow [44].

#### 4.1.1. Impact of mesenchymal stem cell treatment on miR-146a

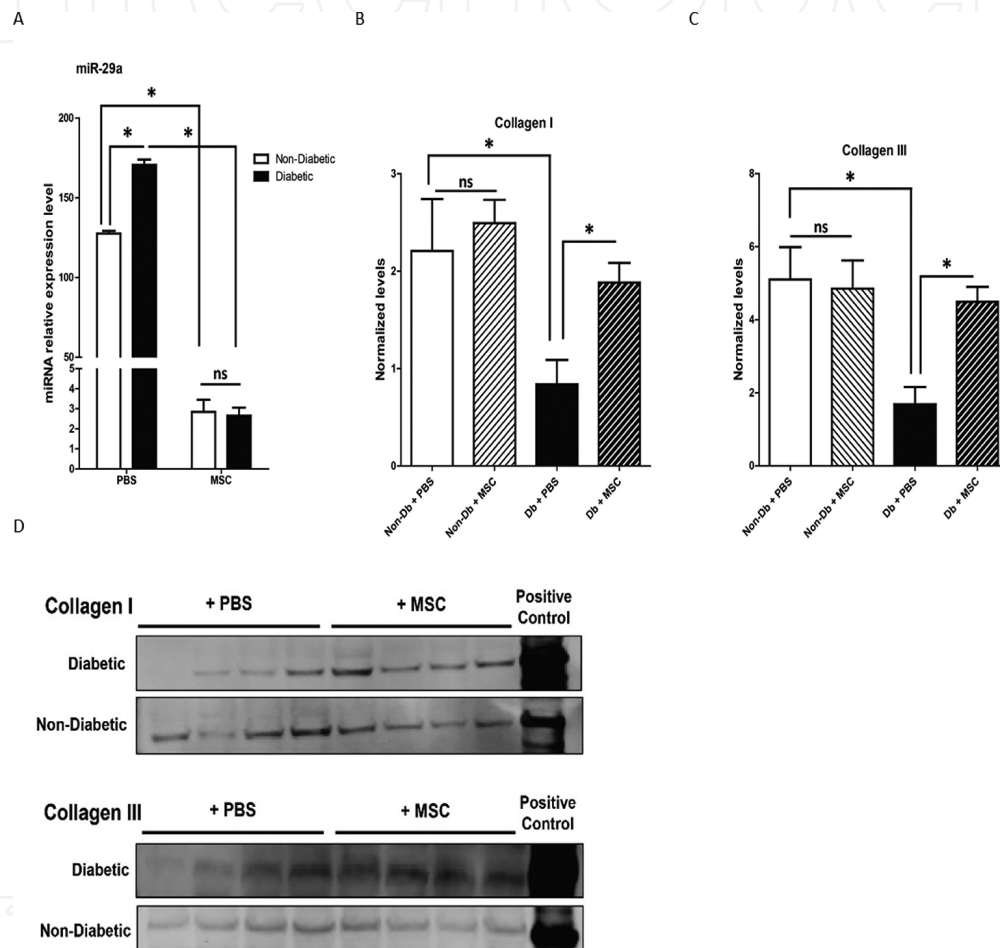
Treatment of diabetic and nondiabetic murine wounds with either MSCs or PBS revealed that treatment of diabetic wounds with MSCs corrected the dysregulated inflammation present in diabetic wounds. Seven days after treatment with MSCs, diabetic murine skin demonstrated more rapid wound healing, a decreased concentration of CD45 positive cells in the periwound tissues, increased expression of miR-146a, and decreased gene expression of IRAK1, TRAF6, NFkB, and the proinflammatory cytokines IL-6 and IL-8/MIP-2 (**Figure 8** and **Figure 9**).



**Figure 9. Impact of MSC treatment on IRAK1, TRAF6, NFkB, IL-6, and MIP2 gene expression.** Real-time PCR demonstrating the impact of treatment with either MSCs or PBS on gene expression of IRAK1 (A), TRAF6 (B), NFkB (C), IL-6 (D), and MIP2 (E) 3 and 7 days after wounding in diabetic and non-diabetic mice. Results are presented as a mean + SEM for each cohort at each time point. Asterisk (\*) indicates  $p < 0.05$  and \*\* indicates  $p < 0.001$ . (Image reproduced from Xu *et al.* [17] with permission of the authors.)

#### 4.1.2. Impact of mesenchymal stem cell treatment on miR-29a

The expedited diabetic wound healing observed after treatment of diabetic skin with MSCs is not solely associated with decreased inflammation. In addition to upregulating gene expression of miR-146a, treatment with MSCs downregulates miR-29a expression in diabetic murine wounds when compared with nondiabetic murine wounds. The downregulation in miR-29a 4 weeks after treatment with MSCs is accompanied by an upregulation in collagen I and collagen III protein content (**Figure 10**).



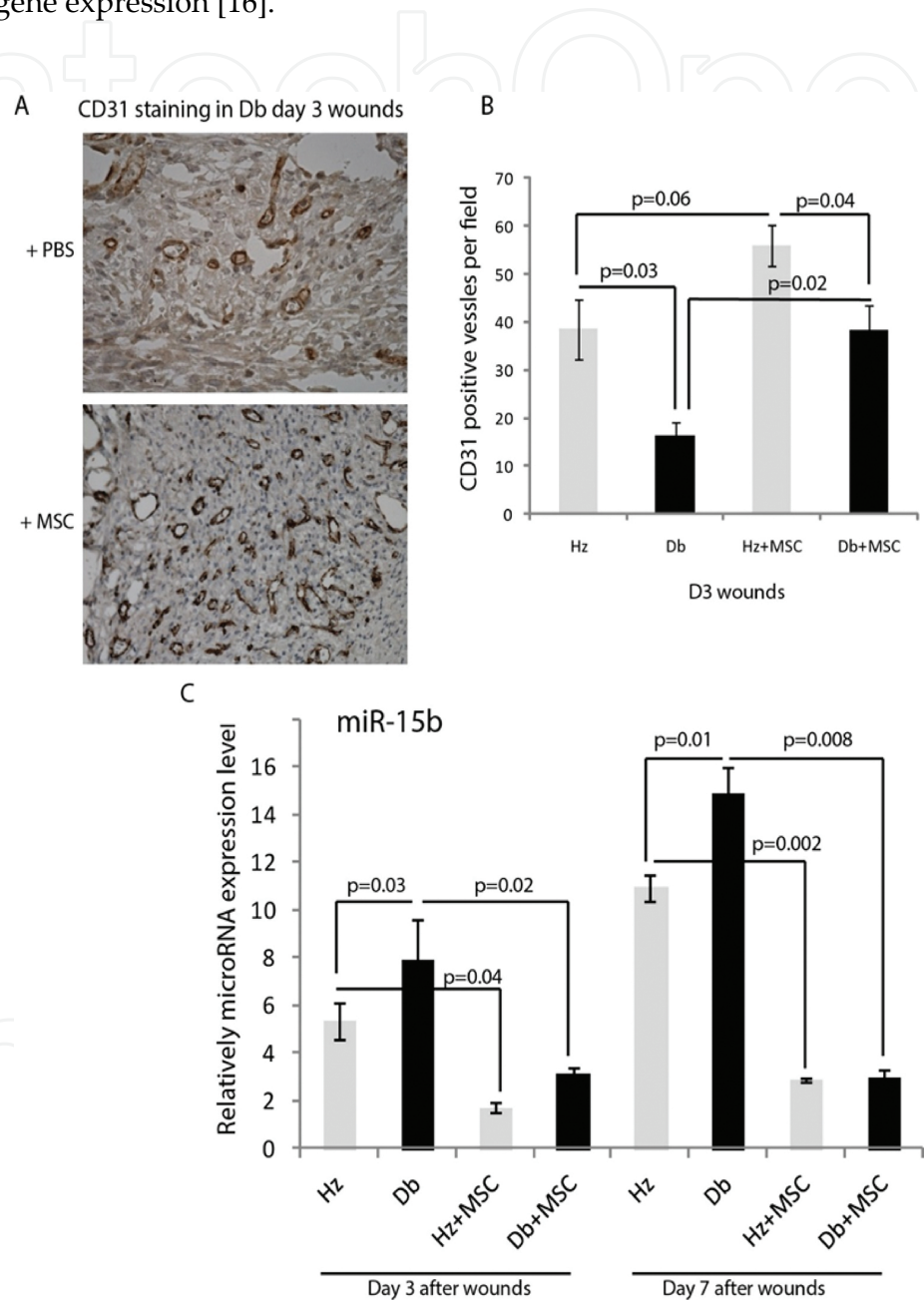
**Figure 10. Impact of MSC treatment on collagen protein and gene expression.** (A) Real-time PCR quantification of miRNA-29a gene expression in diabetic and non-diabetic murine skin 28 days after treatment with MSCs or PBS. Quantification of (B) Collagen I and (C) Collagen III protein levels in diabetic skin and non-diabetic skin 28 days after treatment with either MSCs or PBS. (D) Western blot depicting the Collagen I and Collagen III protein content in diabetic and non-diabetic wounds 28 days after treatment with either MSCs or PBS. Results are presented as a mean + SEM for each cohort at each time point. Asterisk (\*) indicates  $p < 0.05$  and \*\* indicates  $p < 0.001$ . (Image reproduced from Zgheib *et al.* [21] with permission of the authors.)

#### 4.1.3. Impact of mesenchymal stem cell treatment on miR-15b

Treatment with MSCs was also successful at correcting the dysregulated miR-15b expression, further contributing to the improved healing of diabetic wounds observed following treatment



with MSCs. Both 3 and 7 days after wounding and treatment with MSCs, diabetic wounds treated with MSCs demonstrated a significant downregulation in miR-15b gene expression when compares to untreated diabetic wounds (**Figure 11**). Additionally, three days after wounding, diabetic wounds treated with MSCs demonstrated significant upregulation in CD31 positive cell sin the wound bed, as well as significant upregulation in HIF-1 $\alpha$ , BCL-2, and VEGF gene expression [16].



**Figure 11. Impact of MSC treatment on angiogenesis and miRNA-15b expression.** (A) CD31 immunostaining in diabetic wounds 3 days after wounding and treatment with either PBS or MSCs. (B) Quantification of CD31 positive cells in diabetic (Db) and non-diabetic (Hz) wounds 3 days after wounding. (C) Real-time PCR quantification of miRNA-15b gene expression 3 and 7 days after wounding in diabetic (Db) and non-diabetic (Hz) skin treated with either PBS or MSCs. Results are presented as a mean + SEM for each cohort at each time point. P-values are included. (Image reproduced from Xu *et al.* [16] with permission of the authors.)

## 4.2. Adverse effects of MSC treatment

Despite the continued emergence of evidence cataloging the benefits of MSCs in the treatment of diabetic wounds, results are also emerging that detail adverse effects regarding the therapeutic use of MSCs [45]. Specifically, Jeong *et al* (2001) describes the development of soft tissue sarcomas at the site of injection during evaluation of the impact of MSC treatment on both diabetic neuropathy and myocardial regeneration after MI [46]. Similarly, after bone marrow transplant that included systemic administration of  $3 \times 10^6$  MSCs, Tolar *et al.* reported 12 out of 17 (70.5%) mice developed soft tissue sarcomas, including ectopic ossicles and extremity sarcomas [47]. In addition to the risk of malignant transformation following administration of MSCs, the immunosuppressive impact of MSCs therapy may place patients at risk of infection; although this has not been observed *in vivo* [48].

## 4.3. The impact of SDF-1 $\alpha$ on diabetic wound healing

The time and resources required to harvest and prepare an adequate number of MSCs for autologous transplant has led to investigation in to additional means of simulating the robust improvement in wound healing seen after treatment with MSCs. In attempting to define the mechanism by which MSCs and stromal progenitor cells improve wound healing in diabetic mice, it was noticed that the improved wound healing was associated with upregulation of stromal cell-derived factor-1 $\alpha$  (SDF-1 $\alpha$ ). SDF-1 $\alpha$  has long been known as a potent chemokine crucial in the migration and localization of stem cells to wounded tissues [7]. Following injury, SDF-1 $\alpha$  expression is upregulated by HIF-1 $\alpha$  via VEGF in response to hypoxia in the injured tissues [49]. However, SDF-1 $\alpha$  is downregulated in diabetic wounds [7, 22]. We have previously shown that overexpression of SDF-1 $\alpha$  in the wound bed is capable of improving the diabetic wound healing impairment [22]. Furthermore, inhibition of SDF-1 $\alpha$  via transfection with a mutant SDF-1 $\alpha$  that binds the CXCR4 receptor without activation further impairs wound closure, increases inflammatory cytokine production and infiltration of inflammatory cells, and further retards angiogenesis [25]. While these studies support SDF-1 $\alpha$  as a key element in mediating the numerous impairments associated with the diabetic wound healing response, there has been no published evaluation of the impact of SDF-1 $\alpha$  treatment on miRNA dysregulation in diabetic skin or wounds.

## 5. Future directions

While we have presented data on the correction of miRNA dysregulation in a diabetic murine model, future directions include extending these explorations to *in vitro* and *in vivo* human models in order to examine the clinical applicability of treating diabetic wounds with either MSCs or SDF-1 $\alpha$ , while also investigating how the duration of diabetes impacts miRNA dysregulation in human skin. Furthermore, in no way is the miRNA dysregulation documented in diabetes limited to miR-146a, miR-29a, and miR-15b [14, 15, 39]. Numerous families of miRNA are known to be integral to the wound healing process, and we have only touched on a few of the key regulators. In addition to further evaluating the role these additional miRNA



play in regulating the diabetic wound healing response, future directions in this field will likely delve into efforts to make autologous MSC treatment safe and economically feasible, the viability of gene therapy, nanoparticle technology, and improved biomaterials [41, 50, 51]. In addition to regulating protein gene expression, gene therapy could be utilized to upregulate beneficial miRNA expression or downregulate detrimental miRNA expression [39].

The burden of disease attributable to diabetic wounds is projected to intensify as the prevalence of diabetes increases worldwide. As detailed here, patients with diabetes suffer from impaired wound healing, with significant dysregulation at nearly every stage of the wound healing response [2]. This diabetic wound healing phenotype is characterized by decreased angiogenesis, impaired leukocyte migration, decreased growth factor production, sustained inflammation, impaired fibroblast function, imbalance of extracellular matrix deposition and remodeling, and delayed wound healing [9]. Central to the development of this diabetic phenotype is the dysregulation of miRNA that regulate inflammation (miR-146a), extracellular matrix composition (miR-29a), and angiogenesis (miR-15b). We have demonstrated that correction of this microRNA dysregulation through treatment with MSCs expedites wound healing and reverses the diabetic phenotype in skin [16–18], and preliminary results suggest a similar effect following treatment with SDF-1 $\alpha$ . Understanding the role that miRNA play in the regulation of wound healing, as well as the numerous ways miRNA are dysregulated in the diabetic state, will be imperative as we strive to develop more effective wound care technologies in the future.

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