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Regulation of impaired angiogenesis in diabetic dermal wound healing by microRNA-26a

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Abstract

Wound healing is a physiological reparative response to injury and a well-orchestrated process that involves hemostasis, cellular migration, proliferation, angiogenesis, extracellular matrix deposition, and wound contraction and re-epithelialization. However, patients with type 2 diabetes mellitus (T2D) are frequently afflicted with impaired wound healing that progresses into chronic wounds or diabetic ulcers, and may lead to complications including limb amputation. Herein, we investigate the potential role of microRNA-26a (miR-26a) in a diabetic model of wound healing. Expression of miR-26a is rapidly induced in response to high glucose in endothelial cells (ECs). Punch skin biopsy wounding of db/db mice revealed increased expression of miR-26a (~3.5-fold) four days post-wounding compared to that of WT mice. Local administration of a miR-26a inhibitor, LNA-anti-miR-26a, induced angiogenesis (up to ~80%), increased granulation tissue thickness (by 2.5-fold) and accelerated wound closure (53% after nine days) compared to scrambled anti-miR controls in db/db mice. These effects were independent of altered M1/M2 macrophage ratios. Mechanistically, inhibition of miR-26a increased its target gene SMAD1 in ECs nine days post-wounding of diabetic mice. In addition, high glucose reduced activity of the SMAD1-3'-UTR. Diabetic dermal wounds treated with LNA-anti-miR-26a had increased expression of ID1, a downstream modulator of SMAD1, and decreased expression of the cell cycle inhibitor p27. These findings establish miR-26a as an important regulator on the progression

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DISCLOSURES

None.

of skin wounds of diabetic mice by specifically regulating the angiogenic response after injury, and demonstrate that neutralization of miR-26a may serve as a novel approach for therapy.

Keywords

microRNA; diabetes; wound healing; angiogenesis

INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disease associated with a great number of vascular complications including impaired wound healing. Microvascular complications associated with T2D may lead to, for example, limb amputation and increased risk of mortality. Chronic non-healing ulcers in diabetic patients localized on pressure points of the foot is a major cause of non-traumatic lower leg amputation.¹ These ulcers occur not only in the later stages of diabetes but are also seen in newly diagnosed patients. Accumulating studies focus on promoting angiogenesis, or the formation of blood vessels from pre-existing ones, to promote wound healing in diabetic mouse models.^{2–5} Progression of angiogenesis is controlled by a balance between proand anti-angiogenic factors. Vascular ECs need to be rapidly activated to migrate to distant sites and proliferate to form new primary capillaries from existing ones in response to an angiogenic stimuli.⁶ Failure to do so may delay tissue repair in an array of pathological or physiological conditions. Growth factors such as vascular endothelial cell growth factor (VEGF), tumor necrosis factor- α (TNF- α), basic fibroblast growth factor (bFGF), or placenta growth factor (PIGF) are potent regulators of angiogenesis. Impaired EC angiogenic responses have been linked to exacerbation of a wide range of disease states including diabetic wound healing.⁷

MicroRNAs (miRs) are small evolutionarily conserved, 20–22 nt, non-coding RNAs capable of repressing gene expression at the posttranscriptional level by base pairing at the 3' untranslated regions (3'-UTRs) of mRNA targets and have been found to regulate a variety of physiological and cellular functions in health and disease.^{8, 9} Several reports have identified miRs in regulating various aspects of the angiogenic response to diverse pathophysiological stimuli. For example, miR-126, miR-130a, miR-210, and the miR-23~miR-27~miR-24 cluster promote pro-angiogenic activity, whereas miR-221/miR-222, miR-92a, and miR-217 inhibit angiogenic activity in ECs.^{10–16} However, only a few examples of miRs in diabetes-associated angiogenic dysfunction have been reported. For example, miR-126 expression was found to be reduced in CD34⁺ PBMCs in response to high glucose, an effect that impaired their proangiogenic capacity. In a mouse model of diabetic skin wounding, miR-200b expression was increased in response to TNF- α , which in turn targeted GATA2 and VEGFR2 to suppress angiogenesis.¹⁷ Finally, miR-503 is upregulated in response to diabetic conditions, including ischemic limbs, and its neutralization increased blood flow and angiogenesis in diabetic mice with ischemia.¹⁸ Although the studies discussed above investigated various aspects of the role of miRNAs in angiogenic dysfunction in diabetes, the functional role of miRNAs in the context of diabetic wound healing and angiogenesis has not been defined.

Herein, we identify a unique role for miR-26a in regulating the progression of diabetic wound healing. Specifically, our findings indicate that miR-26a expression is induced in wounds of diabetic mice. Neutralization of miR-26a effectively promoted wound closure and was associated with favorable phenotype features including increased granulation tissue, induction of SMAD1 signaling in ECs, and markedly enhanced angiogenesis. We further identify novel cell-specific functional effects of miR-26a that may underlie these observations. Collectively, these findings indicate that therapeutic inhibition of miR-26a may provide a promising treatment for diabetic subjects with impaired dermal wound healing.

MATERIALS AND METHODS

Cell culture and transfection

Human umbilical vein endothelial cells (HUVECs) (cc-2159; Lonza, Walkersville, MD) were cultured in growth media EGM®-2 (cc-3162; Lonza Walkersville, MD). Human dermal microvascular endothelial cells (HMVECs) (cc-2543; Lonza, Walkersville, MD) were cultured in growth media EGM®-2-MV (cc-3202; Lonza Walkersville, MD). Human dermal fibroblasts HDF (cc-2511; Lonza, Walkersville, MD) were cultured in growth media FGM®-2 (cc-3122; Lonza Walkersville, MD). Cells passaged less than five times were used for all experiments. VEGF-A was obtained from R&D Systems. D-Glucose and mannitol were obtained from Sigma. For transfection studies, HUVECs were plated at 50,000 cells/well and cultured overnight before being transfected with Lipofectamine™ 2000 transfection reagent (Invitrogen), following manufacturer's instructions. MiRNA negative controls, and miR-26a inhibitor (AM17100; Ambion, Inc.) were transfected in HUVECs at 100 nM concentration except where indicated. Cy™3 labeled-negative control #1 (AM17120; Ambion) was transfected in parallel to assess transfection efficiency which was >90%. HMVECs and HDFs were cultured with 20mM mannitol or D-Glucose for 72 hours as indicated. For reporter studies, HUVECs were plated (50,000/well) in triplicate on a 12-well plate, grown to 70–80% confluency, and transfected with 800 ng of the indicated reporter constructs and 200 ng β-galactosidase (β-gal) expression plasmids. Transfected cells were collected in 200 μl Reporter Lysis Buffer (Promega). The activity of luciferase and β-gal were measured. Each reading of luciferase activity was normalized to the β-gal activity read for the same lysate.

Real-time qPCR

HUVECs were suspended in TRIzol® reagent (Invitrogen) and total RNA and microRNA was isolated using Trizol® reagent (Invitrogen) per manufacturers instructions. Reverse transcriptions were performed by using miScript Reverse Transcription Kit from Qiagen (218061). Either QuantiTect SYBR Green RT-PCR Kit (204243) or miScript SYBR Green PCR Kit (218073) from Qiagen was used for quantitative real-time qPCR analysis with the Mx3000P Real-time PCR system (Stratagene) following the manufacturer's instructions. Gene-specific primers were used to detect mouse Smad1, ID1, p27, VEGF, Yml, Mgl2, Arg1, Mrc2, Fizz, IL6, IL12, IL1β and NOS2. To amplify mature miRNA sequences, miScript primer assays for Hs_RN5S1_1 (MS00007574), Hs_miR-26a_1 (MS00006559)

from Qiagen were used. Samples were normalized to endogenous 5S RNA (human). Fold changes were calculated by $2^{-\Delta\Delta Ct}$ method.

Chemotaxis assays

Migration assay was performed using ChemTX multiwell system (Neuro probe Inc, MD) with 5 mm pore size and 96 well format. HUVECs transfected with miR-26a inhibitor, or non-specific negative controls were cultured for 72 hours before being plated on the upper compartment of the multiwell plate to assess migration. Lower compartments were filled with EBM-2 medium containing VEGF (R&D Systems). The number of cells migrating to the lower chamber was counted using a hemocytometer after 16 hours. Three technical replicated were used per condition and studies were validated in at least 2 independent experiments.

Western Blots Analysis

HUVECs transfected with miR-26a mimic, miR-26a inhibitor, or non-specific negative controls were cultured for 72 hours. Total cellular protein was isolated by RIPA buffer (50 mM Tris-HCL pH 7.4, 150 mM NaCl, 1%NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (Roche). Cell or tissue debris was removed by centrifugation at 12000rpm for 10 min. Lysates were separated by 8% or 10% SDSPAGE gels, transferred to PVDF membranes (Bio-Rad). Protein quantification was performed using the BCA kit (Thermo Scientific) and cellular lysates were subjected to Western blotting using antibodies against SMAD1 (Cell Signaling), EZH2(Cell Signaling), and β -actin (Cell Signaling). HRP-conjugated goat anti-rabbit or mouse antibody (Santacruz) was used at 1:5000 dilution. ECL assay was performed per manufacturers instructions (RPN2132; GE Healthcare).

Mast cell (MC) staining/Toluidine staining

In brief, sections were deparaffinized and then hydrated in distilled water. Sections were stained with toluidine blue working solution (Sigma, St. Louis, MO) solution for 2–3 minutes and then washed in distilled water. Sections were then briefly dehydrated in 95% and 100% alcohol, air-dried overnight and then analyzed. Intact (nondegranulated) MC were purple in color and contained characteristic granules, while degranulated MC were magenta/violet in color and were devoid of granules. Using the 10x objective, a “hot spot” was chosen (visual field with higher accumulation of MC). The 40x objective was then used to count the number of total, degranulated and nondegranulated cells per visual field (high power field, hpf). The same was done for “non-hot spot” areas. The average for total, degranulated and non-degranulated MC was then calculated. The analysis was performed by two blinded and independent reviewers.

Masson's Trichrome Stain

The manufacturer's protocol (American Mastertech Kit, ATMS Inc., Lodi, CA) was followed. In brief, the sections were deparaffinized and then serially hydrated in ethanol. Sections were stained in Bouin's fluid at room temperature overnight. Next day, sections were washed and then briefly stained in Weigert's hematoxylin solution. With intermittent

rinses, the sections were incubated in Biebrich Scarlet-Acid Fuchsin, phosphomolybdic/phosphotungstic acid, aniline blue, and lastly 1% acetic acid. Sections were dehydrated and coverslips were applied. The 10× objective was used to identify the wound edge and collagen content (blue appearance) was scored with a value of 1 – 5. The analysis was performed by two blinded and independent reviewers.

Peripheral Blood Mononuclear Cells (PBMCs)—Whole blood harvested from WT and db/db mice was collected into EDTA tubes. Blood was then layered onto Ficoll-Paque solution (GE Healthcare, 17-1440-02) and PBMCs were isolated per manufacturers instructions.

In vivo miR-26a inhibition and mouse experiments

Animal protocols were approved by the Laboratory Animal Care at Harvard Medical School. For diabetic wound healing, male, 8–10 weeks old, db/db mice (Jackson) were used to inject intradermally either scrambled control LNA-anti-miR or LNA-anti-miR-26a (Exiqon, Inc) at 50µM (0.63mg/kg) 48h and 24h prior to surgery. On day 0, dorsal fullthickness skin wounds (1cm²) were generated and covered with semi-occlusive dressing (Tegaderm). Images of the wounds were immediately acquired after surgery (day 0) and on days 4 and 9 following the removal of the Tegaderm dressing. Mice were euthanized 9 days post-surgery and the 1×1 cm² sections of skin surrounding the wound were excised down to fascia. Angiogenesis in wound tissue was analyzed by mouse CD31 staining and isolectin B4 (B-1205; Vector Laboratories, Inc) of the paraffin embedded wound sections as we previously described.¹⁹ Wound contraction was calculated using a previously described method²⁰. Granulation tissue thickness was measured on day 10 using H&E stained sections obtained from the center of the wound. Granulation tissue thickness will be defined as the distance of intact tissue from the bottom of the epidermis to the top of the subcutaneous fat layer and will quantified using Image J. Fluorescent images were acquired by Olympus Fluoview FV1000 confocal microscope.

Statistics

Data are presented as mean ± SEM. All *in vitro* experiments are representative of 3 independent experiments. Data were subjected to Student's t-test and *P*<0.05 was considered statistically significant.

RESULTS

MiR-26a is increased in response to diabetic stimuli in ECs

To explore if miR-26a expression is altered in response to diabetic stimuli in ECs, we first examined miR-26a expression in ECs treated with D-glucose. MiR-26a expression was increased by 1.8- and 2.3-fold in response to 3 hrs and 24 hrs, respectively, of D-glucose in HUVECs and 1.2- and 2.2-fold HMVECs in response to 3 hrs and 24 hrs, respectively, of D-glucose treatment compared to mannitol treated cells (Fig. 1A). In addition, 3 hrs of 10mM D-glucose treatment increased miR-26a expression by 1.9 fold (Supplemental Fig. 1E). We also examined miR-26a expression in the wounds of db/db mice. Similar to our *in vitro* findings, miR-26a expression was 3.5- and 1.5-fold higher in db/db mice compared to WT

mice at 4 and 9 days post-wounding, respectively (Fig. 1B). Interestingly, although miR-26a expression did not change significantly on days 4 and 9 post-wounding in WT mice compared to the basal miR-26a expression measured on day 0 (Fig. 1C), its expression increased by 3.2 and 1.6-fold on days 4 and 9 post-wounding, respectively (Fig. 1D). These data suggest that miR-26a may be involved in the earlier pathogenesis of endothelial dysfunction and angiogenesis in response to diabetic stimuli in dermal wound healing.

Inhibition of miR-26a promotes wound healing and angiogenesis in vivo

To understand the role of miR-26a in diabetic dermal wound healing, we neutralized miR-26a expression in db/db mice by direct local intradermal injection of 50 μ M of anti-miR-26a-LNA twice followed by punch biopsy wounding of the skin on the dorsal surface of the mice (Fig. 2A). As previously established by others, diabetic wound healing in mice is significantly impaired compared to non-diabetic wounds^{7, 21, 22, 23, 24}. MiR-26a expression was effectively suppressed by >99% ($p < 0.001$) in the dermal wounds 3 days and 12 days post-injection corresponding to days 0 and 9 of wounding (Fig. 2B) and resulted in a 2.5-fold increase in granulation tissue thickness over control injections after 9 days (Fig. 2C). Remarkably, therapeutic inhibition of miR-26a potently increased wound closure by 53% (Fig. 2D), an effect associated with the induction of robust angiogenesis as demonstrated by increased staining for CD31-positive cells by 1.6-fold and for isolectin-positive cells by 1.8-fold compared to non-specific, scrambled anti-miR controls (Fig. 2E). Collectively these data indicate that inhibition of miR-26a strongly promotes diabetic dermal wound healing and angiogenesis.

MiR-26a deficiency rescues impaired growth and migration in ECs but not in fibroblasts

Dermal wound healing involves a coordinating effort by several cell types including endothelial cells, fibroblasts, keratinocytes, and leukocytes²⁵. To explore the functional role of miR-26a in specific cell types relevant to wound healing, we inhibited the expression of miR-26a in human dermal microvascular endothelial cells (HMVECs), human umbilical vein endothelial cells (HUVECs), and human dermal fibroblasts (HDFs) in the presence and absence of a diabetic stimuli. Interestingly, under basal conditions, stimulation of HUVECs and HMVECs with glucose decreased growth and migration (Supplemental Figure 1A–D) while inhibition of miR-26a rescued impaired growth and migration in the presence of high glucose in HUVECs (Supplemental Figure 1A and 1B) and in the presence of 10mM or 20mM of mannitol or D-Glucose in HMVECs (Fig. 3A and 3B and Supplemental Fig. 1C and 1D). Fibroblasts stimulated with high glucose showed significantly decreased growth and migration in response to NS or miR-26a inhibitor. However, there were minimal effects of anti-miR-26a on fibroblast or keratinocyte growth or migration exclusively in the presence of high glucose compared to the NS inhibitor. (Fig. 3C and 3D and Supplemental Fig. 1F and 1G). In addition, we observed only modest changes in smooth muscle α -actin (SMA- α -actin) and collagen deposition while there was no significant effect on wound contraction in the diabetic dermal wounds in response to miR-26a neutralization (Fig. 3G and Supplemental Fig. 2). Taken together, these data suggest that miR-26a inhibition may mediate cell-specific functional effects in response to diabetic stimuli.

MiR-26a targets SMAD1 signaling in ECs

We have demonstrated that miR-26a exerts its effects on EC growth and angiogenesis through the regulation of the BMP/SMAD1/Id1 signaling pathway.¹⁹ Interestingly, glucose stimulation in ECs lead to the induction of the SMAD1 3'-UTR, an effect completely abolished upon mutation of the 2 miR-26a binding sites (Fig. 4A). Silencing of endogenous SMAD1 expression using SMAD1 siRNA, significantly decreased anti-miR-26a-mediated EC growth and tube formation suggesting that SMAD1 is necessary to mediate angiogenic effects in response to miR-26a inhibition (Fig. 4B and Supplemental Fig. 3). Overexpression of miR-26a significantly decreased matrigel tube formation both under mannitol and D-Glucose treated ECs. (Supplemental Fig. 3). To further examine miR-26a regulation of SMAD1 signaling in the context of diabetic wound healing, we evaluated SMAD1 and its targets ID1 and p27 expression in wounds of db/db mice by local delivery of LNA-anti-miR-miR-26a. As shown in Fig. 4C, both SMAD1 and its target gene ID1 expression were significantly increased, whereas the cell cycle progression gene p27 expression was significantly reduced at the mRNA level. We did not observe an effect of miR-26a neutralization on VEGF expression (Supplemental Fig. 4). Furthermore SMAD1 expression was increased at the protein level by ~ 2.2 fold (Fig. 4D) and it co-localized with CD31-positive cells in the wounds of db/db mice by confocal immunofluorescence staining (Fig. 4E). Collectively, these results indicate that miR-26a targets SMAD1 signaling in diabetic dermal wounds and that miR-26a neutralization restores EC growth and angiogenic function.

Neutralization of miR-26a has no effect on M1 and M2 macrophages or mast cells in diabetic dermal wounds

Macrophages play an important role in mediating dermal wound healing. For example, depletion of macrophages delayed re-epithelialization, reduced collagen deposition, and impaired angiogenesis.^{26–28} Interestingly, we observed a significant decrease in both miR-26a and M1 macrophage marker expression in PBMCs of db/db mice compared to WT mice, whereas the expression of Ym1, an M2 macrophage marker, was significantly increased in the db/db mice compared to the WT mice 9 days post-wounding (Supplemental Fig. 5). To explore whether inhibition of miR-26a in diabetic wounds effect the classically activated pro-inflammatory M1 or the alternatively-activated M2 macrophage subset, we studied the expression markers of these macrophage in wounds of diabetic mice. However, there was no significant effect of miR-26a neutralization on gene expression profiles of M1 or M2 markers (Fig. 5A). In addition, we did not observe a switch from M1 to M2 phenotype by evaluating M1 marker CCR7 and the M2 marker CD206 by immunostaining (Fig. 5B). Similarly, toluidine blue staining for mast cells demonstrated no differences in the accumulation of mast cells or their degranulation in response to local neutralization of miR-26a compared to NS Ctrl (Supplemental Fig. 6). Collectively, these data suggest that while inhibition of miR-26a promotes wound closure, its effects are independent of alteration of M1/M2 polarization or mast cell accumulation in diabetic wounds.

DISCUSSION

Approximately 1 in 4 diabetic subjects will develop a foot ulcer sometime in their lifetime and over two-thirds of these will develop into non-healing wounds despite medical therapy.^{29, 30} The normal homeostatic response to wound healing occurs in progressive phases including inflammation, granulation tissue and angiogenesis, and extracellular matrix remodeling; however, in diabetes, an excessive inflammatory response and decreased angiogenesis contribute to the impaired healing response. Herein, we find that miR-26a expression is increased in response to diabetic wound injury and demonstrate that local neutralization of miR-26a promotes wound healing by inducing angiogenesis in diabetic mice, in part, through the BMP/SMAD1 signaling pathway.

There are a number of cell types such as platelets, keratinocytes, fibroblasts, endothelial, epithelial, and immune cells that orchestrate the complex process of wound healing in response to cytokines and growth factors²⁵. Of these cell types, ECs play a central role in angiogenesis where in response to angiogenic stimuli such as VEGF bFGF, or BMPs they migrate and proliferate to form new blood vessels.^{31, 32} Indeed, diabetic dermal wounds exhibit reduced angiogenesis and therapeutic enhancement of angiogenesis, for example in response to topical angiogenic growth factors, can facilitate and accelerate wound healing in diabetes.^{33, 34, 35} Our studies indicate that diabetic stimuli (e.g. high glucose) markedly increase miR-26a expression in ECs (Fig. 1A). Interestingly, we showed that inhibition of miR-26a rescued impaired growth and migration in the presence of high-glucose in HMVECs and HUVECs. Our findings indicate that inhibition of miR-26a increases growth and migration of HDFs under basal conditions, which is in accordance with recent findings reported by Li, X. et al., albeit in a different type of fibroblasts (lung)³⁶. However, our findings also indicate that there was no effect of anti-miR-26a on dermal fibroblast growth or migration under diabetic conditions (Fig. 3). The lack of functional effects of anti-miR-26a in these cells may be due to the markedly reduced expression of SMAD1 observed in these cells compared to ECs (Supplemental Fig. 7), or that other potential gene(s) not targeted by miR-26a may be important, particularly under diabetic conditions. Furthermore, the miR-26a neutralization in diabetic wounds did not alter the gene expression profiles of M1 proinflammatory or M2 alternatively activated (anti-inflammatory) macrophage markers in diabetic wounds (Fig. 5). Moreover, in response to miR-26a inhibition, there were modest effects on the presence of myofibroblasts in diabetic mice as quantified by SM- α -actin immunostaining (Fig. 3G). Interestingly, cell culture medium from HMVECs transfected with anti-miR-26a and transferred to dermal fibroblasts significantly improved fibroblast cell migration, whereas there were no effects on keratinocytes (Supplemental Fig. 1H and 1I). These data suggest that paracrine effects from ECs with miR-26a inhibition may contribute to dermal fibroblast migration and the more modest effects observed on fibroblasts in vivo. These results highlight that neutralization of miR-26a may primarily regulate ECs and the angiogenic phase of wound healing under diabetic conditions. Collectively, these data strongly implicate that local inhibition of miR-26a induces rapid angiogenesis as the dominant mechanism for the increased granulation tissue thickness and wound closure observed.

Our findings also demonstrated that miR-26a inhibition in diabetic wounds enhanced BMP/SMAD1 signaling by de-repressing its target gene SMAD1 and, in turn, its downstream regulator ID1, a helix–loop–helix transcription factor that lacks a basic DNA-binding domain and can stimulates EC migration and growth.^{37–39} Indeed, in response to miR-26a neutralization, the increased SMAD1 expression co-localized with CD31-positive endothelial cells in diabetic wounds compared to wounds treated with the non-specific LNA-anti-miRs (Fig. 4D). Moreover, the increased expression of ID1 was associated with decreased expression of the cell cycle inhibitor p27 in diabetic wounds in response to miR-26a inhibition. In contrast, our findings revealed that another known miR-26a target EZH2⁴⁰ was not differentially regulated by miR-26a in the wounds of the diabetic mice (data not shown). These findings highlight the growing appreciation that miRNA functional effects are also dictated by cellular context, an effect that is often magnified by discrepancies between primary cells and transformed/tumor cell lines. Future studies will be required to address the relative contribution of other miR-26a targets identified in other cell types to diabetic wound healing.

Accumulating studies suggest a handful of miRNAs may be dysregulated and participate in different pathophysiological phases of diabetic wound healing.⁴¹ For example, miR-155, a miRNA known to functionally regulate diverse aspects of the immune response, recently was found to be induced in diabetic wounds in mice.⁴² Deficiency of miR-155 led to a reduced inflammatory response and improved wound closure, an effect associated with increased expression of miR-155 target genes, BCL6, RhoA, SHIP1, and FIZZ1.⁴² In addition, expression of the members of the miR-99 family were found to be reduced in diabetic wounds and overexpression of this miR family reduced PI3K/Akt signaling and migration and proliferation of keratinocytes, implicating their potential role in the later phase of re-epithelialization⁴³. While the expression of a different miRNA, miR-21, was induced late (day 8) in dermal wounds of diabetic mice and miR-21 overexpression increased fibroblast migration in vitro, the role of altering miR-21 expression in diabetic wound healing remains unknown.⁴¹ In addition, the proinflammatory cytokine TNF- α increased expression of miR-200b in diabetic wounds, an effect that decreased GATA2 and VEGFR2 and altered the angiogenic response; conversely, miR-200b deficiency attenuated TNF- α 's effects and promoted angiogenesis.¹⁷ Finally, a recent examination of human subjects with type 2 diabetes revealed distinct circulating miRNA patterns in the presence of chronic wounds⁴⁴. Interestingly, miR-26a was among the top profiled miRNAs that increased in the chronic diabetic wound group by array-based screening of plasma samples. Taken together, these examples, coupled with the miR-26a-mediated angiogenic effects observed in our study, subserve the notion that miRNAs exhibit distinct and phase-specific effects during the response to diabetic wound healing.

In summary, our findings establish that miR-26a expression is increased in response to diabetic wound injury and that neutralization of miR-26a confers favorable wound healing properties predominantly mediated by effects on angiogenesis. The miR-26a-mediated effects were associated with increased pro-angiogenic BMP/SMAD1-ID1 signaling in ECs and robust functional effects on diabetic ECs, but not dermal fibroblasts. Furthermore, miR-26a inhibition had no effect of altering inflammatory ratio of M1/M2 macrophages in diabetic wounds highlighting that miRNA-mediated control of the angiogenic phase is

sufficient to promote wound healing. Collectively, these findings provide insights to the regulation of angiogenesis in diabetic wound healing by miRNAs and provide new therapeutic targets and strategies to ameliorate complications from diabetic wounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- . Expression of miR-26a is induced in response to high glucose in endothelial cells and post-wounding in the skin of diabetic db/db mice.
- . Local inhibition of miR-26a promotes angiogenesis and dermal wound healing in db/db mice by increasing endothelial SMAD1 expression.
- . Local inhibition of miR-26a had no effect on dermal leukocyte accumulation.
- . MiR-26a may be important in the regulation of the angiogenic response in diabetic wound healing.

Fig. 1. MiR-26a expression is increased in response to diabetic stimuli in vitro and in vivo
 (A) ECs were cultured in the absence or presence of 20 mM D-glucose and harvested for qPCR. (B–D) WT and db/db mice underwent punch-biopsy wounding of the dorsal skin, and wounds were collected for qPCR analyses for miR-26a on the indicated days post-wounding. (B) MiR-26a expression is shown on the indicated days post-wounding in wounds of WT and db/db mice. (C) MiR-26a expression in wounds of the WT mice. (D) MiR-26a expression in the wounds of db/db mice. Data show mean $\pm$ SEM, $n=3$. *, $P < 0.05$.

Figure 2. Local anti-miR-26a-LNA delivery to db/db mice promotes wound healing
 (A) After two local injections in mice of LNA-anti-miR-26a (MiR-26a_i) or scrambled nonspecific control LNA-anti-miRs (NS_i) (n = 11–12 per group), mice underwent dorsal skin wounding. (B–E) Wound analyses included: miR-26a expression quantified by RT-qPCR (B), granulation tissue thickness (GTT) (C), wound closure areas (D), and confocal immunofluorescence staining for CD31 and isolectin (E). Scale bars, 125 μ m (C) and 5mm (D) and 100 μ m (E). All data represent means $\pm$ s.e.m. * P < 0.05, *** P < 0.001

Figure 3. MiR-26a deficiency rescues impaired growth and migration in ECs, but not dermal fibroblasts in response to high glucose
 (A–D) HMVECs and HDFs were transfected with miR negative control (NS_i) or miR-26a inhibitor (miR-26a_i) and cultured in the absence or presence of 20 mM D-glucose. (A,C) Cells were subjected to cell growth assays *P < 0.05, **P < 0.01; (B,D) Cells were subject to migration assay. (E) After two local injections of LNA-anti-miR-26a, db/db mice were wounded by punch biopsy on the upper back and the wounds were collected on day 9 for SM-α-actin by immunostaining. Scale bar 100 μm. Results are representative of n = 4–6 replicates per group. All data represent means ± s.e.m.

Fig. 4. MiR-26a targets SMAD1 signaling in diabetic ECs and skin wounds

(A) Luciferase activity of SMAD1-3'UTR and SMAD1-MUT (harboring 2 mutated miR-26a binding sites) normalized to β -gal was quantified in HUVECs stimulated with 20mM mannitol or glucose for 24 hours ($n = 3$ experiments). $*P < 0.05$. Results are representative of $n = 3$ replicates per group and 3 independent experiments. (B) HUVECs were transfected with a control or SMAD1 siRNA in the absence or presence of miR negative control (NS_i) or miR-26a inhibitor (miR-26a_i). ECs were then subjected to growth assay. $*P < 0.05$. (C–E) After two local injections, wounds from db/db mice were harvested

9 days post-wounding followed by: (C) RT-qPCR for quantitating the expression of SMAD1, ID1 and p27; (D) Western analysis using antibodies to SMAD1 and β -actin; and (E) immunostaining analysis for the quantification of the number of cells co-staining for SMAD1 and CD31. All data represent means $\pm$ s.e.m. *P < 0.05.

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Fig. 5. Neutralization of miR-26a has no effect on M1 and M2 macrophage accumulation in diabetic dermal wounds

After two local injections in mice of LNAanti-miR-26a (MiR-26a_i) or scrambled non-specific control LNA-antimiRs (NS_i) (n = 11–12 per group), mice underwent dorsal skin wounding. (A–B) Wound analyses included: (A) Expression of M1 and M2 macrophage markers quantified by RT-qPCR; and (B) confocal immunofluorescence staining for CCR7 (red), CD68 (green) and DAPI (blue) for M1 and CD206 (red), CD68 (green) and DAPI (blue) for M2 macrophages. Scale bar 100 μ m. All data represent means $\pm$ s.e.m.