MicroRNA-466 and microRNA-200 increase endothelial permeability in hyperglycemia by targeting Claudin-5

Marisa Kujawa,1,3 Megan O’Meara,1,3 Hainan Li,1,3 Liping Xu,1 Sai Pranathi Meda Venkata,1 Huong Nguyen,1 Morgan Minjares,1 Kezhong Zhang,2 and Jie-Mei Wang1,2

1Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy and Health Sciences, Wayne State University, Detroit, MI, USA; 2Centers for Molecular Medicine and Genetics, School of Medicine, Wayne State University, Detroit, MI, USA

Endothelial cell (EC) permeability is essential to vascular homeostasis in diabetes. MicroRNAs are critical gene regulators whose roles in the EC permeability have yet to be characterized. This study aims to examine the change in cell permeability induced by miR-200 and miR-466 in ECs. Human aortic ECs and dermal microvascular ECs from healthy subjects and type 2 diabetic patients were used. Our in vitro experiments unveiled higher expressions of miR-200 family members and miR-466 in diabetic ECs and in healthy ECs when exposed to high glucose. Overexpression of both miR-200 and miR-466 significantly increased EC permeability through transcriptional suppression of Claudin-5, the cell tight junction protein, by directly binding to its 3’ untranslated region. In a mouse model of chronic hyperglycemia mimicking type 2 diabetes in humans (db/db mice), the delayed closure rate of a full-thickness excisional wound was partly rescued by topical application of the miR-200 inhibitor. The topical application of both miR-200 and miR-466 inhibitors exhibited improved efficacy in accelerating wound closure compared with the topical application of miR-200 inhibitor alone. Our study demonstrated the potentially effective approach of miR-200/miR-466 cocktail inhibition to restore vascular integrity and tissue repair in hyperglycemia.

INTRODUCTION
Wound healing is a complex process that consists of multiple molecular and cellular activities. It can be divided into three overlapping phases: inflammatory phase, proliferative phase, and remodeling phase.1 The initial inflammatory response is characterized by the burst of inflammatory cytokines resulting in capillary vasodilation and leakage.7 This transient increase of blood flow and vascular permeability not only allows for inflammatory cell infiltration but also facilitates endothelial sprouting from the existing vascular bed, namely angiogenesis. Angiogenesis is a vital process required for tissue repair. It is necessary to deliver nutrients and oxygen to the wound, support granulation tissue formation, and reconstruct skin structures. In diabetes, this insufficient angiogenesis fails to produce enough capillaries to maintain critical nutrient supply to tissue deposition. Thus, it results in a chronically unhealed wound, mostly seen in the lower limbs, known as diabetic foot ulcers (DFUs).

DFUs have created a significant socioeconomic burden.3 Approximately 15% of diabetic patients with DFUs will eventually go through extremity amputation.7 Multiple factors have contributed to this devastating situation, but one crucial common pathology that occurs early in tissue injury in diabetes is prolonged vascular hyperpermeability. It disturbs the transition from the inflammatory response to the proliferation phase, diminishing angiogenesis and granulation tissue formation,5 because it causes excessive tissue edema and consequently exacerbates the poor delivery of nutrients and oxygen. This prolonged vascular hyperpermeability has been shown to be attributed to the impaired endothelial barrier function1,2 because endothelial cells (ECs) are frequently prone to diabetes-related damage such as hyperglycemia, dyslipidemia, and the proinflammatory state.7 Therefore, understanding how EC barrier function alters under hyperglycemia is critical to developing treatment protocols for wound healing in diabetes.

Tight junctions are important cellular structures crucial to EC permeability.9 Claudins, occludin, and junctional adhesion molecules constitute approximately 20% of the total junctional proteins in the ECs.9,10 Claudin-5 is the major claudin expressed in ECs and is considered vital to EC permeability.11 However, exactly how Claudin-5 is regulated under hyperglycemia remains unknown. Recent investigations targeting dysregulated vascular permeability or angiogenesis using growth factor or cellular interventions have shown promising potential in treating DFUs.12,13 However, skin wound healing is composed of multiple dynamic processes. Inhibiting/promoting one pathway does not necessarily benefit the whole
miRs are small non-coding RNAs, typically 21–25 nt in length. Since its discovery over 30 years ago, the knowledge of miR has been evolving at a fast pace. miRs are now recognized as major players in almost every biological process in eukaryotic organisms, and some are highly conserved across species. In most cases, miRs interact with the 3′ untranslated region (3′ UTR) of target mRNAs to facilitate mRNA degradation and/or to induce translational repression. While numerous studies are investigating miR-mRNA or miR-protein target, relatively few studies of miR-miR interactions or co-actions have been reported. A previous study showed that mouse miR-466(a/b/c/d/e/f/g/h/I, together with miR-467, miR-669, and miR-297, which are located in chromosome 1p36 and chromosome 12p13, respectively) is essential to test molecules that modulate multiple processes in the skin. In fact, several reports have shown concomitant alterations of miR-200 and miR-466 families in a number of settings in rodents and humans. The miR-200 family has five mature miR members in both humans and mice with single-base differences. These five members are encoded from two transcripts that encode the miR-141/miR-200a cluster and the miR-200b/miR-200c/miR-429 cluster, which are located in chromosome 1p36 and chromosome 12p13, respectively. Furthermore, miR-200 family members are upregulated in hyperglycemia,21 and the therapeutic potential of targeting miR-200 family members, predominantly miR-200b/c, in treating diabetes-related vascular disorders has been tested. The miR-200 family, which encodes the miR-141/miR-200a cluster and the miR-200b/miR-200c/miR-429 cluster, which are located in chromosome 1p36 and chromosome 12p13, respectively, is essential. The miR-466 family has one more miR member in humans, while in mice, it has many single-base different family members. miR-466a/b/c/d/e/f/g/h/i, together with miR-467, miR-669, and miR-297, are known as chromosome 2 miR cluster. miR-466 has been reported to participate in the suppression of tumor progression and antiviral defense. However, for both miR-200 and miR-466, it remains unclear whether these two miRs regulate EC permeability.

This study aimed to determine whether miR-200 and miR-466 have a regulatory role in EC permeability and to identify the underlying molecular mechanisms through which miR-466 and miR-200 modulate EC permeability. We also explored the efficacy of cocktail therapy targeting these two miRs in skin wound healing in hyperglycemia. We hypothesized that concurrent upregulation of miR-466 and miR-200 under hyperglycemia increases EC permeability by targeting Claudin-5. Inhibition of these two miRs can restore vascular integrity and pace wound healing. To test this hypothesis, we determined whether high glucose increases miR-466 and miR-200. Next, miR-466 or miR-200 were manipulated to determine whether cell permeability was affected. We also identified Claudin-5 as the direct target of miR-466 and miR-200 in the regulation of cell permeability. In a full-thickness excisional skin wound model, to mimic the skin wound healing in T2D in humans, we used a mouse model of leptin receptor-deficient mice (BKS.Cg-m+/−/Lepr(db)/J, db/db) and their healthy littermates (BKS.Cg-m+/−/Lepr(+/)), lean, db/+). These animals developed hyperglycemia, obesity, dyslipidemia, and insulin resistance as early as 6–8 weeks of age. A full-thickness standard-size excisional wound was created on the dorsal skin. We then tested the impact of miR-466 on wound closure, especially during the early phase of wound healing. The findings from this study provide novel insights into the regulatory roles of miR-466 and miR-200 in endothelial permeability and the dynamic control over the healing course of diabetic skin wounds when miR-466/200 cocktail therapy is implemented.

RESULTS
The upregulation of miR-200 and miR-466 increases EC permeability under hyperglycemic conditions
Using a fluorescein isothiocyanate (FITC)-Dextran Transwell assay, we tested the permeability of the monolayer formed by ECs from healthy donors and T2D donors to determine whether hyperglycemia affects EC permeability. Our results showed a higher permeability of diabetic human aortic ECs (HAECs) than healthy HAECs (Figure 1A), suggesting impaired EC layer integrity in diabetic individuals. Some of the healthy ECs were exposed to 25 mM glucose for 72 h before the permeability test. The healthy ECs exposed to endothelial growth media-2 (EGM-2) supplied with 20 mM mannitol and 5 mM glucose served as control. Consistent with our findings in diabetic ECs, high glucose exposure in vitro elevated the monolayer permeability of healthy ECs (Figure 1B), replicating the impaired cell function in diabetic ECs. Interestingly, we discovered that miR-466 and miR-200 family members, including miR-200a/b/c, miR-141, and miR-429 (stem-loop structure of each miR containing mature sequences obtained from miRbase.org, Release 22.1, are shown in Figure 1C), were increased in ECs from T2D donors (Figure 1D). To test whether the upregulation is due to the high glucose, healthy ECs were exposed to 25 mM glucose for 72 h to mimic hyperglycemia. The treatment of 20 mM mannitol and 5 mM glucose served as control. We found that the levels of miR-200 family members and miR-466 were increased upon high glucose exposure (Figure 1E). Among miR-200 family members, miR-200b and miR-200c showed the most marked changes. To test whether miR-200 or miR-466 contributes to the increased endothelial permeability, healthy ECs were transfected with miR-200 or miR-466 precursors. Real-time PCR tests have confirmed the upregulation of miR-200 family members or miR-466 upon the precursor transfection (Figure 1F). The results suggest that miR-466 alone drives the increased EC permeability and addition of miR-200 does not lead to any synergistic effect when measuring permeability with the FITC-Dextran Transwell assay.

Both miR-466 and miR-200 inhibit EC permeability by directly suppressing Claudin-5
Several molecules have shown potential roles in regulating EC permeability, including vascular endothelial (VE)-cadherin (gene symbol
CDH5), Connexin43 (gene symbol GJA1), and Claudin-5 (gene symbol CLDN5), were on both miR-466 and miR-200 target maps based on databases, including Pictar and TargetScan. We detected protein levels of these molecules in HAECs from healthy and T2D donors. Our results indicated that Claudin-5 was significantly increased in ECs from T2D donors, while Connexin43 and VE-cadherin were comparable to HAECs from healthy donors (Figure 2A).

Because human dermal microvascular ECs (HDMECs) were close to the anatomic location of ECs in the skin, we cultured HDMECs and tested their EC permeability and miR changes. Unlike HAECs, diabetic HDMECs possessed modestly lower Claudin-5 protein levels than healthy HDMECs (Figure 2B, p = 0.137). There was a remarkably suppressed expression of Claudin-5 protein levels in healthy ECs transfected with miR-200 or miR-466 precursors compared to scrambled controls (Figure 2C).

Using a luciferase reporter carrying CLDN5 mRNA 3' UTR, we observed a significant reduction in luciferase activity by the co-transfection of either precursor of miR-200 or miR-466 with luciferase reporter (Figure 2D). Furthermore, we transfected the healthy HAECs with small interfering RNA (siRNA) against Claudin-5 (CLDN5 siRNA) before the miR-200 inhibitor or miR-466 inhibitor transfection. The EC permeability assay suggested that the decreased EC permeability induced by
miR-200 or miR-466 inhibition was blunted by knocking down Claudin-5 (Figure 3B). In HDMECs, we found that the EC permeability could be increased by high glucose (25 mM) conditions (Figure 3C) or knocking down Claudin-5 using siRNA (Figure 3D). The inhibition of the miR-200 and miR-466 by each of their inhibitor transfection was confirmed by real-time PCRs (Figures 3E and 3F). In the meantime, we used western blot analysis to detect Claudin-5 protein expressions in HDMECs upon high glucose treatment. Our results indicated that Claudin-5 protein levels were significantly reduced under high glucose conditions (Figure 3G). Furthermore, the inhibition of miR-466, but not miR-200, was able to restore Claudin-5 protein levels (Figure 3G). In this experiment, we also...
confirmed that the CLDN5 siRNA knocked down Claudin-5 protein expressions by >90% (Figure 3G). Next, we examined the EC permeability in HDMECs transfected with either miR-200 inhibitor, miR-466 inhibitor, or both miR inhibitors, with or without simultaneous transfection of CLDN5 siRNA. Our results demonstrated that the reduction of EC permeability by miR-200 inhibitor (Figure 3H) or miR-400 inhibitor (Figure 3I) was reversed by simultaneously knocking down Claudin-5. HDMECs treated with both miR-200 and miR-466 inhibitors (each at half-dose) demonstrated a trend of reducing EC permeability which was diminished by CLDN5 siRNA (Figure 3J).

Both miR-200 and miR-466 inhibit EC migration, but only miR-200 inhibits EC network formation in vitro
The angiogenic activities of ECs, including migration, network formation, and proliferation, were tested in healthy ECs with miR-200 and/or miR-466 overexpression. Our results suggested that both upregulation of miR-200 or miR-466 dampened EC migration (Figure 4A), but only miR-200 was able to suppress the network formation (Figure 4B). The cell proliferation was not affected by miR-200 or miR-466 (Figure 4C).

Sequential application of miR-466 and/or miR-200 inhibitors rescue wound healing in animals with T2D
The efficacy of miR-200 and miR-466 were tested in a full-thickness excisional cutaneous wound model in animals with T2D. We tested the effectiveness of miR-466 inhibitor at 100 nM every other day in treating diabetic wounds. A moderate acceleration in wound closure was observed in the early inflammatory response phase. However, the overall time course of wound closure was comparable to that of the wounds receiving the control treatment (Figures 5A and 5B). In another set of experiments, we treated the diabetic wounds with a half-dose of miR-466 inhibitor (50 nM) and a half-dose of miR-200 inhibitor (50 nM) for the first 6 days only, then with a full dose of miR-200 inhibitor (100 nM) for the whole healing course (Figure 5C). Real-time PCRs suggested that the miR inhibitors had significantly inhibited the miR-200 and/or miR-466 levels in wound tissues (Figures 5D and 5E). The wound closure of the diabetic wounds receiving miR-200 inhibitor or control treatment was also recorded. Our results demonstrated that the miR-466 inhibitor on the first 6 days significantly enhanced the efficacy of the miR-200 inhibitor alone (Figures 5F and 5G). Furthermore, the histological analysis indicated no significant increase in capillary density formation induced by miR-466 inhibitor as suggested by CD31 staining (Figure 5H). MiR-200 inhibitor increased capillary density, and a short-term combination of miR-466 inhibitor amplified this effect. In fact, wounds receiving both miR-200 and miR-466 inhibitors demonstrated the highest capillary density among all of the groups (Figures 5H and 5I). In addition, the wound Claudin-5 levels were increased by either the miR-466 inhibitor alone or both the miR-200 + miR-466 inhibitors (Figures 5J and 5K). Despite this, the closure pace of miR-200- and miR-466-treated wounds in diabetic mice was slower than that of healthy mice (Figures 5F and 5G), suggesting that other defects hinder the wound healing in miR-200- and miR-466-treated wounds.

DISCUSSION
This study aimed to investigate the roles of miR-200 and miR-466 in vascular permeability in diabetes. Our primary finding was that the upregulation of miR-200 and miR-466 resulted in the increase in EC permeability through targeting Claudin-5 under hyperglycemic conditions (Figure 6). In animals with hyperglycemia, the efficacy of miR-200 inhibitor on skin wound healing could be enhanced by co-administration of the miR-466 inhibitor at the early phase of injury. The study adds new knowledge for the synergistic regulation of miRs on vascular permeability and offers valuable data on the pre-clinical testing of miR cocktail therapies based on the dynamic needs for wound healing.

Different organisms can present various members of the miR family. It is speculated that the genome positions of the miRs affect the capacity of the target gene or gene cluster silencing. This is not the case for these two miRs. miR-200 and miR-466 are from different chromosomes locations in humans and mice and their mature sequences did not show similarity. However, reports have shown the simultaneous changes in miR-200 and miR-466 regardless of the model system. One typical example is that miR-200 and miR-466 are both sodium tonicity-response miRs. To date, it is not clear how hyperglycemic conditions lead to the simultaneous upregulation of these two miRs. Our data indicated that not only osmotic pressure...
but also high glucose per se induced miR-200 and miR-466 because the osmotic pressure was held comparable in high glucose (25 mM) and normal glucose (5 mM) treatments (Figure 1E). A recent genome-wide study observed a reduction in Dicer, an enzyme that produces mature miRs from precursor miRs, caused by sodium hyperosmotic challenge in gill filaments. Similarly, we reported that Dicer transcript and protein levels were reduced in animal models of hyperglycemia. In both scenarios, miR biogenesis should have been suppressed due to deficient Dicer processing. This could not explain the observations that many miRs, including miR-200 or miR-466, are upregulated. We have also shown that insufficient decay of precursor miRs by inositol requiring enzyme 1 in diabetes contributed to the upregulation of a subset of miRs, including miR-466 and miR-200 family members, which could be a working mechanism for the upregulation of miR-200 and miR-466 in hyperglycemia. In both scenarios, miR biogenesis should have been suppressed due to deficient Dicer processing. This could not explain the observations that many miRs, including miR-200 or miR-466, are upregulated. We have also shown that insufficient decay of precursor miRs by inositol requiring enzyme 1 in diabetes contributed to the upregulation of a subset of miRs, including miR-466 and miR-200 family members, which could be a working mechanism for the upregulation of miR-200 and miR-466 in hyperglycemia to be tested in the future. Our study indicated that miR-466 overexpression decreased miR-200b/c levels, which added another layer of complexity in determining miR-miR interactions. Nonetheless, the connection between these two miR families remains elusive and warrants further investigations.

It is known that ECs of different vascular beds demonstrate phenotypic differences despite their similar angiogenic behaviors in response to vasoactive agents. In our experiments, the macrovascular ECs (HAECs) from diabetic donors expressed higher levels of Claudin-5 (Figure 2A), while microvascular ECs (HDMECs) expressed modestly lower levels of Claudin-5 (Figure 2B). We are unsure whether this discrepancy was due to the different comorbidities of the diabetic donors since there was only limited disease information available. However, in HAECs and HDMECs, miR-200 and miR-466 both inhibit Claudin-5 protein expression and increase EC permeability. Microvascular ECs may rely more on Claudin-5, compared to the other ECs, as knockdown of Claudin-5 in HDMECs causes a significant increase in permeability (Figure 3D).

The miR-466 inhibition is more potent in rescuing Claudin-5 and reducing EC permeability than miR-200 (Figures 3G–3I). It is likely that miR-466 inhibition reduces EC permeability through multiple direct or indirect mechanisms, an interesting question to be elucidated for future investigation.

Wound healing involves a finely orchestrated, self-limiting series of cellular and molecular events, resulting in transient increases in vascular permeability and angiogenesis. Increased vascular permeability is a natural consequence of inflammation upon wounding and allows more cytokine extravasation and leukocyte recruitment. The tissue injury and the cytokine extravasation also induce a vigorous angiogenesis response—that is, the growth of leaky capillaries from the existing vasculature. Despite their highly permeable nature, these newly formed vessels help support granulation tissue formation and re-epithelialization, which rebuild the dermis structure. However, excessive and prolonged vascular permeability creates an edemic and inflammatory environment, disturbing the transition...
of wound tissue from the proliferative phase to the remodeling phase. Our in vitro data indicated that miR-200 overexpression augmented EC network formation and migration (Figures 4A and 4B). In contrast, miR-466 overexpression suppressed EC migration but did not inhibit EC network formation, suggesting that miR-200 is more potent in inhibiting angiogenesis than miR-466. However, miR-466 is more potent in increasing EC permeability than miR-200 (Figure 1G). Simultaneous treatment of both miRs did not increase EC permeability any further (Figure 1G). This is important because timely and self-limiting control over vascular permeability is critical for wound healing. MiR-200 inhibition has been shown to increase the expression of vascular endothelial growth factor receptor 2 (VEGF/VEGFR2), which promotes local angiogenesis. However, the randomized clinical trials using VEGF as a proangiogenic agent to treat diabetic wounds have not lived up to expectations, and clinical efficacy remains uncertain. Increased tissue edema due to vascular hyperpermeability seems to be one of the contributing factors to the compromised effectiveness. In the present study, we tested whether controlling vascular permeability by adding miRs that control EC permeability could improve the efficacy. In the early phase of normal wound healing, there is a transient decrease in miR-200 levels in keratinocytes and dermal wound ECs, allowing cell proliferation and migration to occur. However, in diabetes, the downregulation of miR-200 was absent. MiR-200 has been proposed as an excellent target in diabetic wounds, due to not only its proinflammatory and anti-angiogenic actions but also its role in compromising keratinocyte and fibroblast functions. The topical delivery of the miR inhibitors to the wound bed affects not only ECs but also several other cell types in the skin, including keratinocytes and fibroblasts. The efficacy of miR inhibitors on accelerating wound closure is likely associated with the combined efforts of the inhibitors on all of the cell types involved in the wounds.

miR-466 has been recognized as a potent suppressor of oncogenes, inhibiting cell proliferation through several molecules. We anticipated that miR-466 inhibitor would show similar efficacy as miR-200 inhibitor. However, the application of miR-466 inhibitor only showed improvement in wound closure during the first 6 days (Figure 5A). We speculated that the constant increase in vascular permeability was not helpful in the proliferative or remodeling phases. In the meantime, miR-200 inhibitor-treated wounds showed an acceleration of wound healing starting from day 14 compared to wounds with the control treatment. The temporary use of miR-466 inhibitor (days 0, 2, 4) enhanced the efficacy of miR-200 inhibitor (Figures 5F and 5G), with the acceleration of wound closure at the beginning (days 2–6) and then in the remodeling phase of wound healing (days 12–20), compared with the control treatment. Furthermore, the miR-200 and miR-466 inhibitor-treated wounds were covered by a slightly keratinized epithelial layer as early as day 16, while other treatment groups had open wet wounds. The number of oligonucleotides loaded onto the wound bed was comparable among all of the treatment groups. Admittedly, the dosing of oligonucleotides needs to be optimized because the wounds receiving miR inhibitors did not heal as fast as the wounds in healthy mice. One more factor to consider was that both miR-200 and miR-466 were reduced to half-dose when they were used together to treat the cells or the wounds, compared with the single miR inhibitor treatment. This may have contributed to the lesser extent of changes in cell permeability or wound healing when compared with the single inhibitor group. It is challenging for us to evaluate this functional outcome because vascular permeability is usually measured in the skin with an intact epidermis. For example, the Miles assay is used to detect the skin accumulation of the intravenously administered tracers (e.g., Evans blue) that bind to albumin after the local administration of stimulants. Another method is the detection of intravenously administrated fluorescein-conjugated dextrans (<70 kDa) using two-photon microscopy attached to the skin. Because our wound surgery created a defect in the dermis and epidermis, it is inaccurate to measure the outcome of the methods mentioned above. However, it would be of great interest in our future studies. Another technique that may limit the efficacy was that the oligonucleotide-transferring mixture was administered through topical dispensation onto the wound bed instead of multi-point injections at wound edges to create minimal perturbation of tissue regeneration. Despite these limitations, the outcome of the animal study suggests a potentially promising approach to using miR cocktail therapies in wound healing.

In summary, our study indicated that the elevation of miR-466 and miR-200 in diabetes damaged EC integrity and increased cell permeability by targeting Claudin-5. This contributed to the tissue edema in...
the early phase of wound healing, which disturbed the healing process. The miR-200/466 system may offer a therapeutic approach for orchestrating vascular permeability homeostasis in normal physiological diseases. Antisense-based therapeutic targeting of miRs such as miR-200/466 may serve as a novel avenue for the treatment of vaso-pathological disorders associated with metabolic syndrome.

MATERIALS AND METHODS

HAEC culture and HDMEC culture

HAECs from healthy donors and patients with T2D were purchased from Lonza (Basel, Switzerland) and maintained in EGM-2 (Lonza) plus 5% fetal bovine serum in 37°C, 5% CO₂. Donor information: healthy donors, n = 5, age 54.27 ± 3.03 years, male:female = 3:2; T2D donors, n = 5, age 60.60 ± 6.31 years, male:female = 3:2. Both healthy ECs and diabetic HAECs underwent 4–7 passage cycles and were grown until they were 70%–90% confluent. HDMECs from healthy donors and patients with T2D were purchased from Lonza and maintained in microvascular EGM-2 (EGM-2 MV, Lonza) in 37°C, 5% CO₂. Donor information: healthy donors, n = 5, age 58.8 ± 1.068 years, male:female = 1:4; T2D donors, n = 3, age 60.67 ± 2.028 years, male:female = 2:1. All of the HDMECs underwent 4–7 passage cycles and were grown until they were 70%–90% confluent.

Real-time PCR analysis

The miRs were extracted using the miRNAeasy Kit (Qiagen, Germantown, MD, USA). The cDNA was synthesized using miRCURY LNA RT Kit (Qiagen). A real-time PCR was performed to determine levels of miR-466 and miR-200 family members, including miR-200a/b/c, miR-141, and miR249, using U6 as the internal control (primer sets from Qiagen). The initial threshold determined cycle threshold (Ct) values, and the relative expression of RNA was calculated by the comparative ΔΔCt method.

Endothelial permeability assay

Endothelial permeability was tested as previously described. ECs were grown to confluence for a minimum of 3 days in the top well of a Transwell filter (0.4 μm, 12-mm diameter, Corning, Corning, NY, USA). Treatment doses and times are as detailed in the figure legends. The iso-thiocyanate-dextran FITC-labeled fluorescent dye (FITC-Dextran, average 10,000 molecular weight, Thermo Fisher Scientific, Waltham, MA, USA) was added to the top chamber of the Transwell for a final concentration of 1 mg/mL. Permeability was measured by detecting the fluorescent intensity of the amount of dye that leaked through the Transwell filter and reached the lower chamber within 2 h. The fluorescence signal was recorded using GloMax Explorer fluorescent reader (excitation 475 nm, emission 495–505).

miR manipulations

The ECs grown to 70%–90% confluence were transfected with 5 nM miR-200b stem-loop precursor (PM10492, stem-loop accession number: miRBase: MI0000342), or 5 nM miR-466 stem-loop precursor (PM18443, stem-loop accession number: miRBase: MI0014157), or 5 nM Pre-miR miRNA Precursor Negative Control (AM17110, all purchased from Thermo Fisher Scientific) by using Lipofectamine RNAiMAX Reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer’s instructions. For the inhibition of miRs, ECs were transfected with miR-200b inhibitor (AM10492, stem-loop accession number: miRBase: MIMAT0000318), or miR466 inhibitor (AM18443, stem-loop accession number: miRBase: MI0014157), or Anti-miR miRNA Inhibitor Negative Control #1 (AM17010, all from Thermo Fisher Scientific) by using Lipofectamine RNAiMAX Reagent. Cells were incubated at 37°C for 48–72 h before being harvested for experiments.

Cell functional tests, including migration, proliferation, and network formation

The ECs were subcultured into the upper chamber of a 24-well Transwell plate at a density of 3 × 10⁴ cells/well in serum-free EGM-2 media. An amount of 600 μL EGM-2 with 10% fetal bovine serum was added to the lower chamber. The cells were then incubated for 16 h. Media in the upper and lower chambers were discarded, and the inserts were washed with PBS twice. The cells remaining in the upper chamber were gently removed by scraping with a cotton sponge and then were rinsed with PBS twice. The ECs were fixed with 10% formalin for 10 min and stained with 0.5% crystal violet for 30 min at room temperature. The insert membrane was then washed with PBS six times until transparent. Images were taken using EVOS FL Imaging System (Thermo Fisher Scientific). The number of cells that migrated to the lower side of the membrane was counted and recorded as the migrated cells per 200 high-power field.

Western blot analysis

Cells were lysed using Cell Lytic MT lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) with Protease Inhibitor Cocktail (1:100 v/v, Thermo Fisher Scientific) for 20 min on ice. The lysate was sonicated at 20% amplitude for 30 s on ice. The protein concentration was determined by Bradford assay using Quick Start™ Bradford 1 X Dye Reagent (Bio-Rad, Hercules, CA, USA). Equal amounts of denatured protein (30 μg) were separated by SDS-PAGE on 10% Tris-glycine
polycrylamide gels and transferred to a 0.45-µm polyvinylidene fluoride membrane (Sigma-Aldrich) together with Chameleon Duo Pretained Protein Ladder (Li-Cor, Lincoln, NE, USA). Immunoblotting was performed by using antibodies directed against each target molecule: Claudin-5 (1:1,000, Invitrogen), Connexin43 (1:1,000, Abcam, Cambridge, UK), and VE-cadherin (1:1,000, R&D, Minneapolis, MN, USA). The housekeeping protein was β-actin (1:10,000, Cell Signaling Technology, Danvers, MA, USA). Secondary antibodies, including IRDye donkey anti-goat IgG (1:20,000), IRDye goat anti-mouse IgG (1:20,000), and IRDye goat anti-rabbit IgG (1:20,000) from Li-Cor were incubated at room temperature for 1 h. For the immunoblotting of two target proteins with close molecular weights on the same membrane, such as Connexin43 and β-actin, we did not strip the membrane between two probing as the secondary antibodies for Connexin43 and β-actin are from different species and are conjugated with different fluorescent dyes. These two secondary antibodies ensure minimum or no signal interference between separate channels in the fluorescent imaging system. Membrane-bound antibody fluorescence signals were detected by Li-Cor Odyssey CLx. Quantitative analysis of protein levels was analyzed with Image Studio Lite version 5.2 (Li-Cor). The target protein expression was divided by β-actin to get the relative abundance (considered as the first delta) in each sample. The mean value of the control group was calculated, and the value of each sample was then divided by the mean value to obtain the fold change (considered as the second delta). The data were presented as the fold change versus the control treatment group.

3' UTR luciferase activity assays
Synthetic oligonucleotides of human Claudin-5 (CLDNS) mRNA 3' UTR (Target Gene Accession: NM_003,277.3) were cloned into a luciferase reporter vector system (SwitchGear, Carlsbad, CA, USA). Healthy ECs were co-transfected with 100 ng CLDNS 3' UTR reporter and 0.1 nmol miR precursors, either miR-200 or miR-466, or 0.1 nmol scramble controls (all purchased from Ambion, Waltham, MA, USA) using DharmaFECT Duo transfection reagent (Dharmacon, Lafayette, CO, USA) according to the manufacturer’s protocol. After 48 h, luciferase activity was measured. A reduced firefly luciferase expression indicates the direct binding of miRs to the cloned target sequence.

Cutaneous wound healing with miR-200 and miR-466 inhibitors in diabetic animals
Male T2D mice (BKS.Cg-m+/- Lepr/db/+), db/db, age 12–14 weeks, blood glucose over 250 mg/dL and their age- and sex-matched nondiabetic healthy littermates (BKS.Cg-m+/- Lepr/db/- lean, db/+), blood glucose 123.80 ± 19.72 mg/dL were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). A full-thickness excisional wound was created on dorsal skin using a 7-mm punch biopsy without damaging the underlying muscles, as we previously described.32,45 For topical delivery of miR inhibitors, anti-miR miRNA inhibitor (Thermo Fisher Scientific), 100 nM miRNA inhibitor in Lipofectamine™ RNAiMAX (Invitrogen) in 50 µL of Opti-MEM were topically placed onto the wound bed immediately after punch. The grouping was as follows: (1) db/db wound with 100 nM scrambled control (anti-miR negative control #1, Ambion); (2) db/db wound with 100 nM miR-200b inhibitor (50 nM hsa-miR-200-5p and 50 nM hsa-miR-200-3p, Ambion); (3) db/db wound with 50 nM miR-466a inhibitor (25 nM mmu-miR-466-5p and 25 nM mmu-miR-466-3p, Ambion) and 50 nM of miR-200b inhibitor on days 0, 2, and 4, followed by 100 nM miR-200b inhibitor for the remaining days; (4) db/+ wound with 100 nM scramble control. Immediately after, wounds were covered with a transparent oxygen-permeable wound dressing (Tegaderm™ film, 3M Medical, Eden Prairie, MN, USA). A freshly prepared miRNA inhibitor was administered on the wound every other day with the change of dressing. Wound closure rates were calculated as percentage closed (y%) = [(area on day0 – open area on dayx)/area on day0] × 100. On day 20 after wounding, wounds and the adjacent skin were collected for CD31 and Claudin-5 immunohistochemistry staining. All of the animal procedures were performed according to Wayne State University Institutional Animal Care and Use Committee (IACUC) guidelines.

Statistical analyses
All of the values are expressed as means ± SDs. For continuous variables that failed Shapiro-Wilk normality tests such as miR expression, protein levels, staining quantifications, and functional assays, the statistical significance of differences between the two groups was determined by the Mann-Whitney U test. When more than two groups of treatments were performed, we used the Kruskal-Wallis test across all of the groups. If significant, we then tested the pairs of our primary interest based on scientific rationale using the Mann-Whitney U test with Benjamini, Krieger, and Yekutieli’s adjustment for multiple comparisons.66 Due to multiple hypothesis testing, these gatekeeping approaches and the adjustments can preserve the alpha spending and control false positive rate inflation (<0.05). The significant differences that came from post hoc comparisons of groups were noted. A value of p < 0.05 was considered statistically significant. All of the statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).

DATA AVAILABILITY STATEMENT
The dataset generated in this study are available from the corresponding author upon reasonable request.

ACKNOWLEDGMENTS
This work was supported in part by NIH/NIDDK R01 DK109036, R01 DK119222, and R01 DK128937 (to J.-M.W.). M.M. was supported by the institutional training grant IMSD at Wayne State University (T32 GM 139807). We thank the Department of Laboratory Animal Research staff at Wayne State University for providing excellent animal care.

AUTHOR CONTRIBUTIONS
M.K. and M.O. conducted the in vitro experiments, collected and analyzed the data, and wrote the manuscript; H.L. conducted the in vitro experiments and the in vivo wound healing experiments and edited the manuscript; L.X. assisted with the experiments for
33. Nagy, J.A., Benjamin, L., Zeng, H., Dvorak, A.M., and Dvorak, H.F. (2008). Vascular permeability, vascular hyperpermeability and angiogenesis. Angiogenesis 11, 109–119. https://doi.org/10.1007/s10456-008-9099-z.

34. Mittal, M., Siddiqui, M.R., Tran, K., Reddy, S.P., and Malik, A.B. (2014). Reactive oxygen species in inflammation and tissue injury. Antioxid. Redox Signal. 20, 1126–1167. https://doi.org/10.1089/ars.2012.5149.

35. Giacca, M., and Zacchigna, S. (2012). VEGF gene therapy: therapeutic angiogenesis in the clinic and beyond. Gene Ther. 19, 622–629. https://doi.org/10.1038/gt.2012.17.

36. Lo, W.Y., Yang, W.K., Peng, C.T., Pai, W.Y., and Wang, H.J. (2018). MicroRNA-200a/200b modulate high glucose-induced endothelial inflammation by targeting O-linked N-acetylglucosamine transferase expression. Front. Physiol. 9, 355. https://doi.org/10.3389/fphys.2018.00355.

37. Zhou, R., Wang, C., Liang, Y., Li, X., and Li, Q. (2019). Anti-miR-200b promotes wound healing by regulating fibroblast functions in a novel mouse model. Acta Biochim. Biophys. Sin. 51, 1049–1055. https://doi.org/10.1093/abbs/gmz091.

38. Colden, M., Dar, A.A., Saini, S., Dahiya, P.V., Shahryari, V., Yamamura, S., Tanaka, Y., Stein, G., Dahiya, R., and Majid, S. (2017). MicroRNA-466 inhibits tumor growth and bone metastasis in prostate cancer by direct regulation of osteogenic transcription factor RUNX2. Cell Death Dis. 8, e2572. https://doi.org/10.1038/cddis.2017.15.

39. Haig, D., and Mainieri, A. (2020). The evolution of imprinted microRNAs and their RNA targets. Genes J. 11, E1038. https://doi.org/10.3390/genes11091038.

40. Malnou, E.C., Umlauf, D., Mouysset, M., and Cavaille, J. (2018). Imprinted MicroRNA gene clusters in the evolution, development, and functions of mammalian placenta. Front. Genet. 9, 706. https://doi.org/10.3389/fgene.2018.00706.

41. Ono, S., Egawa, G., and Kabashima, K. (2017). Regulation of blood vascular permeability in the skin. Inflamm. Regen. 37, 11. https://doi.org/10.1186/s41232-017-0042-9.

42. Jiang, R., Cai, J., Zhu, Z., Chen, D., Wang, J., Wang, Q., Teng, Y., Huang, Y., Tao, M., Xia, A., et al. (2014). Hypoxic trophoblast HMGB1 induces endothelial cell hyperpermeability via the TRL-4/caveolin-1 pathway. J. Immunol. 193, 5000–5012. https://doi.org/10.4049/jimmunol.1303445.

43. Zhou, L., Zhang, H.H., Chen, N., Zhang, Z.B., Liu, M., Dai, L.F., Wang, J.M., Jiang, Y.W., and Wu, Y. (2019). Clinical features of 54 cases of leukoencephalopathy with vanishing white matter disease in children. Zhonghua Er Ke Za Zhi 57, 837–843. https://doi.org/10.3760/cma.j.issn.0578-1310.2019.11.005.

44. Monaghan-Benson, E., and Burridge, K. (2009). The regulation of vascular endothelial growth factor-induced microvascular permeability requires Rac and reactive oxygen species. J. Biol. Chem. 284, 25602–25611. https://doi.org/10.1074/jbc.M109. 009894.

45. Lang, J., Xu, F.J., Ge, W.K., Liu, B.Y., Zhang, N., Sun, Y.H., Wang, J.M., Wang, M.X., Xie, N., Fang, X.Z., et al. (2019). Greatly enhanced performance of AlGaN-based deep ultraviolet light emitting diodes by introducing a polarization modulated electron blocking layer. Opt Express 27, A1458–A1466. https://doi.org/10.1364/OE.27.0A1458.

46. Benjamin, Y., Krieger, A.M., and Yekutieli, D. (2006). Adaptive linear step-up procedures that control the false discovery rate. Biometrika 93, 491–507. https://doi.org/10.1093/biomet/93.3.491.