Transient High Glucose Causes Persistent Vascular Dysfunction and Delayed Wound Healing by the DNMT1-Mediated Ang-1/NF-κB Pathway

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The progression of diabetic complications does not halt despite the termination of hyperglycemia, suggesting a metabolic memory phenomenon. However, whether metabolic memory exists in and affects the healing of diabetic wounds, as well as the underlying molecular mechanisms, remain unclear. In this study, we found that wound healing was delayed, and angiogenesis was decreased in mice with diabetes despite the normalization of glycemic control. Thus, we hypothesized that transient hyperglycemic spikes may be a risk factor for diabetic wound healing. We showed that transient hyperglycemia caused persistent damage to the vascular endothelium. Transient hyperglycemia directly upregulated DNMT1 expression, leading to the hypermethylation of Ang-1 and reduced Ang-1 expression, which in turn induced long-lasting activation of NF-κB and subsequent endothelial dysfunction. An in vivo study further showed that inhibition of DNMT1 promoted angiogenesis and accelerated diabetic wound healing by regulating the Ang-1/NF-κB signaling pathway. These results highlight the dramatic and long-lasting effects of transient hyperglycemic spikes on wound healing and suggest that DNMT1 is a target for diabetic vascular complications.


INTRODUCTION

As a serious complication of diabetes, refractory wounds significantly impair the QOL for patients and lead to skin ulcers, nonhealing diabetic foot, and eventual limb amputation (Dumville et al., 2012; Edmonds, 2012; Rüttermann et al., 2013). Current therapies, such as GF treatment (Falanga et al., 1992) and bioactive dressings (Dinh and Veves, 2006; Mustoe et al., 2006), have not been fully efficacious, and there is limited understanding as to which patients with diabetes are susceptible to the development of chronic, nonhealing wound (Dinh et al., 2012) and why therapeutic strategies are effective in some patients but not in others.

Strong epidemiological evidence has revealed that diabetic complications progress unimpeded even after glycemic control is pharmacologically achieved (Diabetes Control and Complications Trial Research Group et al., 1993; Holman et al., 2008; Turner et al., 1999). This harmful phenomenon is known as metabolic memory (MM), is supported by laboratory evidence (Kowluru, 2003; Li et al., 2006; Roy et al., 1990), and is defined as permanent abnormalities in cell functions in response to initial exposure to hyperglycemia despite normalization of glycemic control (Lee et al., 2016). Clinically, we have observed that there are no obvious improvements in delayed wound healing even when glycemic control is pharmacologically achieved in patients with diabetes. Thus, we hypothesized that transient hyperglycemic spikes exert a long-lasting effect on diabetic wound healing. However, whether the phenomenon of MM actually exists in and affects diabetic wound healing, as well as the mechanism underlying MM-induced endothelial dysfunction during wound healing, are still unclear.

The underlying molecular mechanisms of diabetic complications may include the involvement of the advanced glycation end products, the excess ROS, and alterations in tissue-wide gene expression patterns (Brownlee, 2005; Giacco and Brownlee, 2010; Pirola et al., 2010). However, the ability to sustain these complications in the absence of hyperglycemia invokes a role for epigenetics in perpetuating these complications through MM. Epigenetic alterations create a persistent change that is stored as memory and passed on to the offspring usually in response to microenvironmental stimuli (Fetita et al., 2006), and these epigenetic alterations may be a key mechanism underlying MM and sustained vascular dysfunction despite the achievement of glycemic control (Brownlee, 2005; Pirola et al., 2010; Reddy and Natarajan, 2011). Recent in vitro investigations into the
epigenome’s role in MM have documented specific hyperglycemia-induced DNA methylation modifications that persist in the MM state (Reddy and Natarajan, 2013; Rodriguez and El-Osta, 2018). Alterations of DNA methylation may become particularly important in the context of MM because only DNA methylation is backed by strong mechanistic support for the heritability of epigenetic changes (Kaufman and Rando, 2010; Maunakea et al., 2010). However, to date, the DNA methylation modifications associated with MM in diabetic refractory wounds have not been clearly studied.

In this study, MM was examined as an important pathogenic factor in diabetic wound healing. We showed that transient hyperglycemia caused persistent damage to the vascular endothelium. Transient hyperglycemia directly upregulated DNMT1 expression, leading to the hypermethylation of Ang-1 and reduced Ang-1 expression, which in turn induced long-lasting activation of NF-kB and subsequent endothelial dysfunction. An in vivo study further showed that the inhibition of DNMT1 promoted angiogenesis and accelerated diabetic wound healing by regulating the Ang-1/NF-kB signaling pathway. Our results implicate MM as an important mechanism in diabetic wound healing and suggest that blocking DNMT1 may have therapeutic value in treating diabetic vascular complications and delayed wound healing.

RESULT

Transient hyperglycemia induces delayed wound healing

Wound healing progressed slowly in the group with diabetes mellitus (DM) and in the group with MM (Figure 1b). The wound closure rate was 30% in the control group, and it was less than 15% in the group with DM and group with MM on day 3. On day 7, the closure rate in the control group reached 60%, whereas it was only 15% and 17% in the group with DM and group with MM, respectively. The wounds in the control group reached complete closure by day 14, whereas there were still 41% and 37% unhealed wound areas in both group with DM and group with MM, respectively (Figure 1c).

Thickened epidermis, abundant infiltration of inflammatory cells, and hemorrhagic scabs were observed in the group with DM and the group with MM (Figure 1d). Moreover, collagen deposition increased, and the collagen fibers were disorderly arranged in the group with DM and the group with MM group (Figure 1e).

Proliferating capillary index, which is reflected by the percentage of CD31-positive microvessels with Ki67-positive endothelial cell nuclei (Figure 1f), was quantified, and the results showed that the proliferating capillary index values were significantly lower in the group with DM and the group with MM than in the control group (Figure 1i). The total number of microvessels (stained with CD31) was calculated, and the results showed that the group with DM and the group with MM had much lower average microvessel densities than the control group (Figure 1j). The microvessel pericyte coverage index was used to determine the percentage of microvessels covered with pericytes and was quantified to evaluate the maturity of the neovasculature (Figure 1g). The microvessel pericyte coverage indexes in the group with DM and the group with MM were observably lower than those in the control group (Figure 1k). In addition, the number of macrophages at the wound site was higher in the group with DM and the group with MM than in the control group (Figure 1h). The expression of IL-1ß, TNF-α, and ICAM-1, cytokines that are closely related to wound healing, markedly increased in the group with DM and the group with MM compared with those in the control group (Figure 1i–n).

Transient hyperglycemia damages the functions of endothelial cells

To simulate the transient hyperglycemia conditions in vitro, human umbilical vein endothelial cells were first incubated in high glucose (HG) for 24 hours and then cultured in normal glucose (NG) for 2 days (MM-1), 4 days (MM-2), and 6 days (MM-3). Human umbilical vein endothelial cells cultured in NG were used as a control, and human umbilical vein endothelial cells cultured in HG alone were used to represent DM without tight glucose control (Figure 2a). In addition, 30 mM mannitol served as an osmotic control (Supplementary Figure S1).

HG treatment significantly increased the permeability of endothelial monolayers to FITC-dextran, which persisted in a normoglycemic environment for the entire 6-day period (Figure 2b). Correspondingly, the transendothelial electrical resistance of endothelial monolayers decreased after HG treatment and remained at this low level after subsequent exposure to NG (Figure 2c). The functional protein E-cadherin was evenly distributed in a continuous pattern along the endothelial intercellular junctions in the control group, and HG treatment caused a substantial loss of cell surface–associated E-cadherin expression. Notably, this decreased expression persisted during subsequent incubation under NG conditions (Figure 2d).

In addition, the ability of endothelial cells to form capillary-like tubes was dramatically reduced after HG treatment (Figure 2e and f), and this reduction persisted for 6 days with subsequent exposure to NG. The adhesion of monocytes to endothelial cells was stimulated by transient hyperglycemia and remained elevated during 6 days of subsequent incubation under NG conditions (Figure 2g). Transient hyperglycemia induced an increase in the expression of the proinflammatory cytokines IL-1ß, TNF-α, and ICAM-1, which persisted for the subsequent 6 days of exposure to NG conditions (Figure 2h–j).

Transient hyperglycemia damages the functions of endothelial cells by inhibiting the expression of Ang-1

Our previous research reported that Ang-1 plays an important role in advanced glycation end product–induced endothelial cell dysfunction (Zhao et al., 2015). Advanced glycation end products are prevalent in the diabetic vasculature and are considered to be an important cause of diabetic vascular complications (Goldin et al., 2006); therefore, we asked whether Ang-1 is implicated in transient hyperglycemia–induced persistent microvascular impairments. The mRNA and protein expression of Ang-1 significantly decreased after exposure to transient HG, and this decrease persisted for 6 days of subsequent exposure to NG conditions. (Figure 3a–c). COMP-Ang-1 (cAng-1), a potent recombinant Ang-1 protein variant (Cho et al., 2005),
revered the transient HG–induced high permeability and low transendothelial electrical resistance of endothelial monolayers (Supplementary Figure S2a and b). Similarly, cAng-1 treatment prevented the substantial loss of cell surface–associated E-cadherin expression that was caused by transient hyperglycemia (Supplementary Figure S2c). Moreover, cAng-1 improved the transient HG–mediated injury to the tube formation ability of endothelial cells (Supplementary Figure S2d and e). In addition, cAng-1 inhibited the transient HG–induced persistent excessive inflammatory conditions, including the increased adhesion of monocytes to endothelial cells (Supplementary Figure S2f) and overexpression of IL-1β, TNF-α, and ICAM-1 (Figure 2g–i).

**Figure 1.** Transient hyperglycemia induces delayed wound healing. (a) Schematic representation of the experimental model in vivo. (b) Representative wound images at d 0 and 14 during the healing process. (c) The wound-healing rate was measured every d and is shown as the percentage of the initial wound area (n = 8 per group). (d, e) Histological changes and collagen deposition in wounds were evaluated by (d) H&E staining and (e) Masson staining. Bar = 100 μm. (f, g) Representative images of double staining of (f) Ki67 and/or CD31 (red for CD31; green for Ki67; blue for nuclei) and (g) α-SMA and/or CD31 (red for CD31; green for α-SMA; blue for nuclei) in skin sections from different experimental groups. Bar = 100 μm. (h) Representative images of staining of macrophages. Bar = 50 μm. (i–k) Quantitative comparison of the (i) PCI, (j) MVD, and (k) MPI in the different groups. (l–n) The expression of inflammatory cytokines, including (l) IL-1β, (m) TNF-α, and (n) ICAM-1 in the different groups (n = 8). *P < 0.05 (compared with Con mice treated with PBS). α-SMA, α-smooth muscle actin; Con, control; d, day; DM, diabetes mellitus; HG, high glucose; MM, metabolic memory; MPI, microvessel pericyte coverage index; MVD, microvessel density; NG, normal glucose; PCI, proliferating capillary index; w, week.
Decreased Ang-1 expression induced by transient hyperglycemia is linked to hypermethylation of the Ang-1 promoter

We next studied how transient hyperglycemia affects the expression of Ang-1. When compared with the methylation levels in endothelial cells cultured under normal conditions, increased methylation was detected in the promoter region of Ang-1 in cells after transient hyperglycemia, and this increase persisted for 6 days of subsequent exposure to NG conditions (Figure 3d and e). Moreover, Ang-1 promoter activity was significantly decreased in transient hyperglycemia–treated endothelial cells, which also persisted for 6 days of subsequent exposure to NG conditions (Figure 3f). Furthermore, treatment of endothelial cells with the methyltransferase inhibitor 5-aza-deoxycytidine (5-Aza), an effective DNA demethylating agent, significantly decreased the methylation...
level of the Ang-1 promoter and resulted in the activation of Ang-1 gene expression (Figure 3g). Conversely, treatment of cells with the methyl donor S-adenosyl-L-methionine, which has been shown to inhibit demethylase activity and induce DNA methylation (Detich et al., 2003), led to upregulated methylation of the Ang-1 promoter (Figure 3h). To further explore whether DNA methylation directly regulates Ang-1 promoter activity, the promoter region of Ang-1 was cloned into a luciferase reporter construct. SssI methylase (methylation of 12 CpGs), HpaII methylase (methylation of two CpGs), and HhaI methylase (methylation of two CpGs) (Lu et al., 2015) were used to clone the fragment, and Ang-1 promoter activity was determined after transfection of endothelial cells with methylated or mock-methylated luciferase constructs. As shown in Figure 3i, methylation inhibited Ang-1 promoter activity in a methylation dose-dependent manner.

**Elevated DNMT1 expression is involved in the transient hyperglycemia–induced decrease in Ang-1 expression**

We demonstrated that aberrant DNA methylation is associated with decreased Ang-1 expression. In this study, we detected the expression of DNMTs, and the results showed that the mRNA and protein expressions of DNMT1 were increased (Figure 4a–c) and the DNMT1 activity was elevated (Figure 4d) in endothelial cells that were subjected...
to HG injury, and the increased status persisted during subsequent incubation under NG conditions. Immunofluorescence staining also showed that DNMT1 was mainly located in the nucleus, and the expression remained high after transient HG treatment (Figure 4e). However, neither the activity nor the expression of DNMT3a nor that of DNMT3b changed in endothelial cells after HG treatment (Supplementary Figure S3). Chromatin immunoprecipitation assay indicated that the binding of DNMT1 to the Ang-1 promoter was enhanced by transient HG treatment, and these changes persisted for 6 days of subsequent incubation at NG levels (Figure 4f).

In addition, the specific DNMT1 inhibitor 5-Aza and small interfering RNA against DNMT1 (siDNMT1) were used to inhibit the expression of DNMT1, and the efficiency of inhibition was confirmed by western blot analysis (Figure 4g). The results showed that siDNMT1 or 5-Aza treatment reversed the decreased mRNA (Figure 4h and i) and protein expression of Ang-1 that was induced by transient HG exposure (Figure 4j and k). Moreover, after the inhibition of DNMT1 expression, the binding of DNMT1 to the Ang-1 promoter was decreased (Figure 4l and m), whereas Ang-1 promoter activity was enhanced even after transient HG treatment (Figure 4n and o).

Figure 4. Elevated DNMT1 expression is involved in the transient hyperglycemia–induced decrease in Ang-1 expression. (a) The mRNA expression of DNMT1 in endothelial cells subjected to different hyperglycemia treatments (n = 6). Quantification of (b, c) the protein expression of DNMT1 (n = 4) and (d) the activity of DNMT1 (n = 6). (e) Immunofluorescence staining showing the expression and cellular localization of DNMT1 in endothelial cells subjected to different hyperglycemia treatments. Bar = 50 μm. (f) ChIP assay showing the binding of DNMT1 to the promoter of Ang-1 in endothelial cells (n = 6). (g) DNMT1 expression was knocked down by siRNA and 5-Aza in endothelial cells, and the efficiency of knockdown was confirmed by western blot analysis (n = 4). (h, i) Relative mRNA expression of Ang-1 in endothelial cells with DNMT1 knockdown under different hyperglycemia conditions (n = 6). (j, k) Western blot analysis showing the expression Ang-1 in endothelial cells with DNMT1 knockdown by (j) siDNMT1 or (k) 5-Aza (n = 4). (l, m) The binding of DNMT1 to the promoter of Ang-1 in endothelial cells after inhibition of DNMT1 (n = 6). (n, o) Luciferase reporter assay showing the Ang-1 promoter activity of endothelial cells after inhibition of DNMT1 (n = 6). *P < 0.05 (compared with Con group). 5-Aza, 5-aza-deoxycytidine; ChIP, chromatin immunoprecipitation; Con, control; DM, diabetes mellitus; MM, metabolic memory; NG, normal glucose; siDNMT1, small interfering RNA against DNMT1; siRNA, small interfering RNA.
Elevated DNMT1 expression is involved in transient hyperglycemia-induced endothelial cell dysfunction

We demonstrated that transient hyperglycemia damages the functions of endothelial cells by inhibiting the expression of Ang-1, whereas DNMT1 directly regulates Ang-1 activation and expression. Thus, we asked whether DNMT1 is involved in transient hyperglycemia-induced endothelial cell dysfunction. As shown in Figure 5a–d, siDNMT1 or 5-Aza treatment reversed the transient HG-induced high permeability and low transendothelial electrical resistance value of endothelial monolayers. Moreover, the addition of 5-Aza or siDNMT1 application largely restored the loss of cell surface-associated E-cadherin expression caused by transient hyperglycemia treatment (Figure 5e and f). In addition, siDNMT1 or 5-Aza treatment improved the transient HG-induced weakened tube formation ability of endothelial cells (Figure 5g–j). In addition, siDNMT1 or 5-Aza treatment significantly suppressed the excessive inflammatory response, including the increased adhesion of monocytes to endothelial cells (Figure 5k and l) and the overexpression of IL-1β, TNF-α, and ICAM-1, caused by transient hyperglycemia treatment (Figure 5m–r).

NF-κB pathway activation is involved in DNMT1/Ang-1 signaling–induced endothelial cell dysfunction

Ang-1 protects endothelial cell function by inhibiting the activation of the NF-κB pathway (Fan et al., 2004; He et al., 2014). Active methylation and increased NF-κB
transactivation play critical roles in the development of diabetic complications, such as nephropathy and retinopathy (Duraisamy et al., 2018; Zhang et al., 2017), suggesting that the effect of DNMT1/Ang-1 signaling on transient hyperglycemia–induced endothelial cell dysfunction involves the NF-κB pathway.

The results showed that the phosphorylation of NF-κB p65 (at Ser536) increased after transient hyperglycemia treatment, and phosphorylated p65 expression remained at this high level after subsequent exposure to NG (Supplementary Figure S4a). Immunofluorescence staining showed that there was a significant increase in the nuclear translocation of the NF-κB subunit p65 in cells after transient hyperglycemia stimulation, and this nuclear distribution pattern persisted during subsequent incubation under NG conditions (Supplementary Figure S4b and c). Moreover, transient hyperglycemia induced an increase in NF-κB luciferase reporter activity, which persisted for the subsequent 6 days of exposure to NG conditions (Supplementary Figure S4d).

We next investigated whether DNMT1 directly affects NF-κB activity. As shown in Supplementary Figure S4e and f, siDNMT1 or 5-Aza significantly decreased the transient hyperglycemia–induced phosphorylation of NF-κB p65. In addition, cells with reduced DNMT1 expression showed reduced nuclear NF-κB p65 expression (Supplementary Figure S4g–i) and reduced NF-κB luciferase reporter activity (Supplementary Figure S4j and l), even in hyperglycemia conditions.

We then examined whether Ang-1 was essential for transient hyperglycemia–induced NF-κB activation. The results showed that transient hyperglycemia–induced phosphorylation of NF-κB p65 (Supplementary Figure S5a), nuclear translocation of NF-κB p65 (Supplementary Figure S5b and c), and increased NF-κB luciferase reporter activity (Supplementary Figure S5d) were markedly reversed by cAng-1 treatment.

Finally, we determined whether blocking the NF-κB pathway (Pierce et al., 1997) attenuated transient hyperglycemia–induced endothelial cell dysfunction. As shown in Supplementary Figure S6a and b, BAY 11-7085 (Sigma-Aldrich, St. Louis, MO) treatment reversed the transient hyperglycemia–induced high permeability and low transendothelial electrical resistance value of endothelial monolayers. BAY 11-7085 also prevented the loss of cell surface–associated E-cadherin expression that was caused by transient hyperglycemia (Supplementary Figure S6c). Moreover, BAY 11-7085 treatment observably inhibited the transient HG–injured tube formation ability of endothelial cells (Supplementary Figure S6d and e) as well as the excessive inflammatory responses (Supplementary Figure S6f–i).

Inhibition of DNMT1 promotes the healing of diabetic wounds

Immunohistochemical staining showed a strong positive expression of DNMT1 in the wounds from the group with DM and the group with MM. In contrast, weak staining for DNMT1 was observed in the wounds from the control group (Supplementary Figure S7b and c). When compared with the expression in the control group, the expression of DNMT1 was significantly increased in the group with DM and the group with MM (Supplementary Figure S7d). Furthermore, the expression of Ang-1 was clearly reduced (Supplementary Figure S7e and f), and the activation of NF-κB was increased in the group with DM and the group with MM when compared with those in the control group (Supplementary Figure S7g).

In addition, the wound closure rate was significantly increased in the group with DM and the group with MM that were treated with either siDNMT1 or 5-Aza (Figure 6a and b). siDNMT1 or 5-Aza also improved the re-epithelialization and revascularization as well as decreased the excessive inflammatory cell infiltration and collagen deposition (Figure 6c). The repressed angiogenic process in the group with DM and the group with MM (Figure 6d and e), such as decreased proliferation of endothelial cells (proliferating microvessels density) (Figure 6f), and the immaturity of the neovasculature (microvessel pericyte coverage index) (Figure 6g), was significantly improved after the inhibition of DNMT1 by siDNMT1 or 5-Aza. The overexpression of inflammatory cytokines in the group with DM and the group with MM were obviously reduced by siDNMT1 or 5-Aza treatment (Figure 6i–k). To evaluate the blood flow perfusion, a laser Doppler imager was used, and results showed that the wound perfusion in the group with DM and the group with MM was much lower than that in the control group. siDNMT1 or 5-Aza treatment significantly increased the wound perfusion in the group with DM and the group with MM (Supplementary Figure S8). In addition, the increased number of macrophages and the increased expression of DNMT1 in the group with DM and the group with MM were reduced after siDNMT1 or 5-Aza treatment (Supplementary Figure S9a–d). More importantly, the decreased expression of Ang-1 (Supplementary Figure S9e–g) and the upregulated phosphorylation of NF-κB p65 (Supplementary Figure S9h) were reversed after the inhibition of DNMT1 by siDNMT1 or 5-Aza.

DISCUSSION

In this study, we found that long-lasting excessive inflammatory reaction and deficiency in angiogenesis is due to continuing injury from exposure to transient hyperglycemic conditions, leading to delayed wound healing through MM. Initial researches have revealed that histone modifications are maintained in the posthyperglycemic environment (Li et al., 2008; Miao et al., 2008). DNA methylation, which has the strongest experimental support for heritability and could contribute to heritable transmission (Maunakea et al., 2010), has recently been associated with MM and the progression of diabetic complications. Using a diabetic zebrafish model, it was found that the reduced fin regeneration caused by hyperglycemia is maintained through the MM, and the global DNA hypomethylation that correlated with aberrant gene expression is the potential mechanism (Olsen et al., 2012). Another study also reported that increased DNA methylation of POLG1 promoter could contribute to the MM phenomenon observed in the progression of diabetic retinopathy (Tewari et al., 2012). However, these studies are limited because they do not have a detailed investigation of how MM alerts DNA methylation and the associated genes expression.
In this study, we found that elevated DNMT1 expression was involved in the sustained dysfunction of endothelial cells caused by MM. As one of DNA methyltransferases, in contrast to DNMT3a and/or DNMT3b, which mediate the establishment of new or de novo DNA methylation patterns (Okano et al., 1999), DNMT1 classically functions as a maintenance DNMT because it conserves the methylation pattern during replication (Robert et al., 2003). Epigenetic modification by DNMT1 leads to various biological consequences related to inflammation, matrix remodeling, and immune regulation (Robertson and Wolffe, 2000). Recently, several lines of evidence have shown that alterations in DNMT1 expression and activity play important roles in diabetic complications (Reddy and Natarajan, 2011), such as retinopathy (Kowluru et al., 2016), nephropathy (Lu et al., 2017), and cardiovascular complications (Costantino et al., 2019).

Figure 6. Inhibition of DNMT1 promotes the healing of diabetic wounds. (a) Representative wound images on d 0 and d 14 during the healing process. (b) The wound-healing rate was quantified and is shown as the percentage of the initial wound area (n = 5 per group). (c) Histological changes and collagen deposition in wounds were evaluated by H&E and Masson staining. Bar = 100 μm. (d, e) Representative images of double staining of (d) Ki67 and/or CD31 and (e) α-SMA and/or CD31 in skin sections. Bar = 50 μm. (f–h) Quantitative comparison of the (g) PCI, (f) MVD, and (h) MPI in the different groups. (i–k) The expression of inflammatory cytokines, including (i) IL-1β, (j) TNF-α, and (k) ICAM-1, in the different groups (n = 10). *P < 0.05 (compared with Con group), †P < 0.05, ‡P < 0.05. 5-Aza, 5-azacytidine; α-SMA, α-smooth muscle actin; Con, control; d, day; DM, diabetes mellitus; MM, metabolic memory; MPI, microvessel pericyte coverage index; MVD, microvessel density; PCI, proliferating capillary index; siDNMT1, small interfering RNA against DNMT1.
In this study, we found that the expression and activity of DNMT1 were elevated in endothelial cells after transient hyperglycemia exposure, and inhibition of DNMT1 reversed the transient hyperglycemia-induced impaired angiogenesis, promoting wound healing, indicating that the increased DNMT1 is the key factor in the refractory wounds caused by MM. In addition, we also found that DNMT1 induces methylation in the Ang-1 promoter, resulting in decreased Ang-1 expression. As a strong endothelial-specific protective factor, Ang-1 promotes the formation of mature and functional microvessels, maintains endothelial integrity, and protects microvessels against plasma leakage (Lee et al., 2003; Yancopoulos et al., 2000). Our previous study demonstrated that Ang-1 protects endothelial cells against advanced glycation end products damage, indicating the potential protective function of Ang-1 in microvascular injury in individuals with diabetes (Zhao et al., 2015). Therefore, DNMT1-mediated Ang-1 hypermethylation served as a key mechanism in the impaired angiogenesis and delayed wound healing caused by MM. To further explore the underlying mechanisms by which DNMT1/Ang-1 signaling influences wound healing, we detected the activation of NF-κB. Previous studies have demonstrated that Ang-1 protects endothelial cells against hyperglycemic damage by inhibiting the activation of the NF-κB pathway (Fan et al., 2004; He et al., 2014), and the increased methylation and NF-κB activation also play important roles in diabetic complications (Duraiasmey et al., 2018; Zhang et al., 2017), suggesting that the NF-κB pathway may be involved in the impaired angiogenesis mediated by DNMT1/Ang-1 signaling. As expected, we found that the activation of NF-κB was increased after transient hyperglycemia, and inhibition of DNMT1 or exogenous application of Ang-1 reversed transient hyperglycemia-induced NF-κB activation, indicating that DNMT1 and/or Ang-1 affects angiogenesis and wound healing process by regulating the NF-κB pathway.

In summary, the observations reported in this study show that transient hyperglycemia causes persistent endothelial dysfunction during subsequent normoglycemia by inducing long-lasting changes in DNA methylation and recruitment of DNMT1 to the Ang-1 promoter, leading to decreased expression of Ang-1 and increased NF-κB activation. Together, these results implicate MM as an important mechanism in delayed wound healing in diabetes and indicate that blocking DNMT1 may have therapeutic value in treating diabetic vascular complications and delayed wound healing.

**MATERIALS AND METHODS**

**Cell culture**

Human umbilical vein endothelial cells were purchased from Lonza (Walkersville, MD) and maintained in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum and antibiotics. Cells at passages 3–5 were used and incubated in a medium containing 30 mM HG or 5 mM NG (Figure 2a) and 30 mM mannitol, which served as an osmotic control.

**Mouse experiments**

**Protocol for mice with diabetes.** The mice were divided into three groups: control group, group with diabetes, and group with MM. Diabetes was induced in the mice with streptozotocin (55 mg/kg; Sigma-Aldrich). In the group with diabetes, the mice were maintained with poor glycemic control (glycated hemoglobin was 10–12%) by receiving 1 unit of insulin (Humulin; Novo Nordisk, Copenhagen, Denmark) every other day for 4 months. In the group with MM, the mice were in poor glycemic control for 2 months, followed by good glycemic control (receiving insulin twice daily for a total of 8 units; glycated hemoglobin was 5–7%) for two additional months. Age-matched normal mice without any treatment served as the control group. The weights of the mice were measured twice a week, the blood glucose was detected once a week (Glucometer Elite; BAYER, Leverkusen, Germany) (Supplementary Figure S7a), and glycated hemoglobin was evaluated every 2 months (Helena Laboratories, Beaumont, TX).

**Induction of cutaneous wounds.** To simulate the biological processes of human wound healing, the splinted murine wound model was used. By splinting the wound, the repair process is then dependent on epithelialization, cellular proliferation, and angiogenesis. Briefly, two 5-mm-thick wounds were made on each side of the dorsum of the mice using a biopsy punch. A silicone splint was placed around the wound with the assistance of adhesive, and the splint was then secured with interrupted sutures (Dunn et al., 2013; Galiano et al., 2004). siDNMT1 or 5-Aza diluted in PBS (30 ng/ml; Li Rui Biological Technology Co., Ltd, Shanghai, China) was applied to the wounds, and PBS was used as a control. All wounds were covered with sterile gauze and bandaged. The experimental solutions were applied to the wounds daily, and the wounds were observed and assessed.

**Data analysis**

All statistical analyses were performed using SPSS software, version 17.0 (SPSS, Chicago, IL). Data in this study are expressed as the mean ± SD. The number of independent replicates of every part of an experiment is represented using the letter n in the figure legend. The differences between experimental groups were compared using the unpaired Student’s t-test or one-way ANOVA followed by post-hoc analysis with the Bonferroni test. *P < 0.05 was considered statistically significant.

**Ethics Statement**

The experiments in this study were approved by the First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China).

**Data availability statement**

Datasets related to this article can be found at [https://data.mendeley.com/datasets/cpd72hjtf3/1](https://data.mendeley.com/datasets/cpd72hjtf3/1), hosted at Mendeley.

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**CONFLICT OF INTEREST**

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS
Conceptualization: BS, SQ; Data Curation: JX, XL; Formal Analysis: JZ, SY, YX; Investigation: JZ, SY, LC, RV, YX; Writing - Original Draft Preparation: JZ, SY

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.10.023.

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