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Targeting epigenetic mechanisms in diabetic wound healing

Aaron den Dekker, PhD¹, Frank M. Davis, MD¹, Steve L. Kunkel, PhD², and Katherine A. Gallagher, MD¹

¹Department of Surgery, University of Michigan, Ann Arbor, MI 48109

²Department of Pathology, University of Michigan, Ann Arbor, MI 48109

Abstract

Impaired wound healing is a major secondary complication of type 2 diabetes that often results in limb loss and disability. Normal tissue repair progresses through discrete phases including hemostasis, inflammation, proliferation, and remodeling. In diabetes, normal progression through these phases is impaired resulting in a sustained inflammatory state and dysfunctional epithelialization in the wound. Due to their plasticity, macrophages play a critical role in the transition from the inflammation phase to the proliferation phase. Diabetes disrupts macrophage function by impairing monocyte recruitment to the wound, reducing phagocytosis, and prohibiting the transition of inflammatory macrophages to an anti-inflammatory state. Diabetes also impedes keratinocyte and fibroblast function during the later phases resulting in impaired epithelialization of the wound. Several recent studies suggest that altered epigenetic regulation of both immune and structural cells in wounds may influence cell phenotypes and healing, particularly in pathologic states, such as diabetes. Specifically, it has been shown that macrophage plasticity during wound repair is partly regulated epigenetically and that diabetes alters this epigenetic regulation and contributes to a sustained inflammatory state. Epigenetic regulation is also known to regulate keratinocyte and fibroblast function during wound repair. In this review, we provide an introduction to the epigenetic mechanisms that regulate tissue repair and highlight recent findings that demonstrate how epigenetic events are altered during the course of diabetic wound healing.

Keywords

Epigenetics; Wound Healing; Type 2 Diabetes

Introduction

Over 30 million people over the age of 18 in the US have diabetes mellitus (1). Additionally, another 86 million live with 'pre-diabetes', a condition that can progress to type 2 diabetes (T2D) (1). The overall prevalence of diabetes continues to increase, thereby contributing to

Corresponding author: Katherine A. Gallagher, MD, Department of Surgery, University of Michigan, 1500 East Medical Center Drive, SPC 5867, Ann Arbor, MI 48109, Phone: (734) 936-5820, Fax: (734) 647-9867, kgallag@med.umich.edu.

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increasingly high healthcare costs (1). Diabetes-related healthcare expenditure was 548 billion USD in 2013, accounting for 11% of the total adult healthcare cost, and is projected to exceed 627 billion USD by 2035 (2). Further, it is estimated that approximately 7 million people go undiagnosed thus delaying disease treatment and management (1). Non-healing wounds that result in lower limb amputation, are among the most common complications associated with diabetes and account for more than 200 billion USD annually in healthcare costs and loss of productivity (3). Importantly, amputation is associated with high mortality rates of 16.7% at 12 months and over 50% at 5 years, rates that are worse than many cancers (4).

Impaired wound healing in diabetes is the result of a combination of factors that promote inflammation and disrupt epithelialization and wound closure. Although this process is complex, it has been shown that neutrophils and macrophages recruited to the site of the wound are critical components of the healing process (5–7). During the normal healing process, macrophages are initially pro-inflammatory and then transition to an anti-inflammatory phenotype where they promote tissue repair and transition to the next phase of healing (6, 8, 9). In diabetic wounds, however, this macrophage transition is altered, causing them to remain in a pro-inflammatory state and prevent wound resolution (10–13). The factors driving the macrophage transition are not fully understood but recent evidence suggests that epigenetics plays a role in regulating macrophage function in both normal and diabetic wound healing (11, 14–17). These processes include DNA methylation of CpG islands, and methylation of histone tails.

Additionally, re-epithelialization is delayed in diabetic wounds due to impaired keratinocyte and fibroblast function brought on by hyperglycemia and accumulation of advanced glycation end-products (AGEs) (18–21) and epigenetic regulation has also been implicated in these processes (22–24).

Given the impact on human health, substantial work has focused on understanding the mechanisms by which the cells involved in the wound healing response are regulated and how these mechanisms are disrupted in the setting of diabetes. In this review, we provide a brief overview of epigenetics and diabetic wound healing and highlight the recent advances related to the convergence of these two fields.

Normal wound healing

Phases of wound healing

Wound healing is a dynamic process that occurs in a programmed series of four phases: hemostasis, inflammation, proliferation, and remodeling (25). These steps occur in a linear forward fashion under normal conditions. Following injury, hemostasis occurs and is characterized by the recruitment of platelets and circulating coagulant factors to the wound site to initiate clotting (26). Concurrent with platelet recruitment, injured cells release damage signaling factors, which activate resident macrophages (27–30), as well as damage associated molecular patterns (DAMPs) (31). Together, these stimulate recruitment of polymorphonuclear neutrophils (PMNs) from the vasculature to defend against pathogens (27, 29, 31). As platelets aggregate to the exposed collagen bed, they secrete cytokines and

growth factors such as platelet-derived growth factor, that promote clotting and serve to further recruit PMNs (32). Once PMNs begin to migrate to the wound, this initiates the inflammation stage.

Neutrophils dominate during the early part of the inflammation phase; however, their presence is short-lived as they start to wane after 24–48 hours (33). Neutrophils release chemokines that are responsible for recruiting circulating monocytes from the peripheral blood to the wound site. Recruited monocytes that differentiate into macrophages and dendritic cells, along with F4/80+ resident tissue macrophages, carry out the critical steps of the inflammatory phase of wound healing (10, 34). However, the specific roles of recruited monocytes/macrophages, dendritic cells, and tissue macrophages in wound healing are unclear (35). Wound macrophages initially display a “classical” activation state, indicated by expression of pro-inflammatory cytokines such as IL-12, IL-1 β , IL-6, TNF α , and iNOS. Pro-inflammatory, or M1-like, macrophages work to clear debris and recruit additional inflammatory cells. As the inflammatory phase progresses, macrophages phagocytose apoptotic cells in the wound and chemokines are released (e.g., CXCL12), promoting the transition from a pro-inflammatory state to one promoting tissue repair and remodeling (36, 37). This occurs as the predominant macrophage phenotype in the wound transitions to an “alternatively activated” state, or M2-like, secreting anti-inflammatory cytokines such as IL-4, IL-10, IL-13, and TGF β (34, 38). *In vivo*, macrophages exist along a spectrum and the distinct phenotypes seen *in vitro* (e.g., M1, M2) are rarely seen (34, 39, 40).

The subsequent proliferation phase is characterized by the recruitment and activation of keratinocytes and fibroblasts. During this phase, growth factors stimulate keratinocytes to reepithelialize the wound (38). During this time, the provisional matrix established by platelets during hemostasis is replaced by granulation tissue. Fibroblasts secrete proteinases and matrix metalloproteases (MMPs) to degrade the provisional matrix while simultaneously secreting collagen and other extracellular matrix (ECM) proteins into the granulation tissue (25).

The final phase of wound healing is the remodeling phase and begins once granulation tissue is present. Here, fibroblasts differentiate into contractile myofibroblasts that contract the wound and the collagen III deposited in the ECM during the proliferation phase is exchanged for collagen I which has a higher tensile strength (41). Once healed, the site is mechanically functional but many structural components, e.g., hair follicles, may not recover and the healed site has, at maximum, ~70% of the original tensile strength (38).

Diabetic foot ulceration and delayed wound healing

The etiology of non-healing diabetic foot ulcerations is multifactorial due to a combination of peripheral neuropathy, peripheral artery disease, and altered immune function (42). These factors work together to predispose the diabetic patient to ulceration and infection. Despite the cause of the wound, it is failure of the wound to heal that is responsible for considerable morbidity and mortality associated with diabetes. Neuropathic edema makes the diabetic foot susceptible to ulceration and infection (43) and ischemia resulting from occlusive arterial disease reduces blood flow (44), both contributing to delayed healing in the diabetic

wound. Additionally, recruitment of endothelial progenitor cells is impaired in diabetes due to reduced nitric oxide (NO) production which, ultimately, results in impaired angiogenesis (45). However, the most direct effects on wound healing come from functional alterations in cells activated by the immune response, including platelets, macrophages, neutrophils, endothelial cells, fibroblasts, and keratinocytes; all contributing to a failure to progress through the normal phases of wound healing.

Platelets

Platelets are key constituents of hemostasis and are responsible for some of the earliest events in response to injury, initially forming a platelet plug. As healing progresses, however, the clot must be degraded in order for re-epithelialization to occur. Sustained high glucose and insulin-resistance causes platelets to release high levels of fibrinogen and plasminogen activator inhibitor 1 (PAI-1) making them more adherent to the vascular endothelium and more likely to aggregate (46, 47). Additionally, under diabetic conditions, platelets have been shown to be less responsive to NO released from the vascular endothelium which normally reduces aggregation at the vessel wall (48). Exacerbating this, defects in insulin signaling cause the vascular endothelium to produce less NO (49, 50). Thus, platelet dysfunction in diabetes contributes to development of micro-vascular disease that is well-established in diabetic patients. Hence, in the setting of wound healing, the platelet phenotype induced by diabetes hinders wound healing by reducing coagulation during hemostasis.

Neutrophils

Neutrophils are important components during the inflammatory phase of wound healing, working to clear pathogens. However, sustained recruitment and activation of neutrophils are associated with chronic, non-healing wounds (51–53). Elevated levels of neutrophil-derived proteases are believed to contribute to persistent inflammation and delayed wound healing (51, 52). In addition to proteases, neutrophils release extracellular traps (NETs) composed of decondensed chromatin lined with cytotoxic proteins to kill microbes (53). It has been shown that the protein responsible for NET formation, peptidyl deiminase 4 (PAD4), is elevated in neutrophils from diabetic mice and humans making them more susceptible to NETosis and potentially contributing to persistent inflammation and tissue damage in diabetes (53).

Monocytes/Macrophages

Monocytes are recruited to the wound site very early during the inflammatory phase where they differentiate into macrophages and dendritic cells. Despite the lack of clarity regarding the *in vivo* definition of these cells, there is substantial evidence that infiltrating monocytes/macrophages are critical for establishing the initial inflammatory phase as well as promoting the transition from a pro-inflammatory to anti-inflammatory environment (6, 8, 11). In diabetic wounds this transition does not occur and macrophages remain in a persistent inflammatory state where they promote the destruction of the surrounding tissue both directly and indirectly by recruiting other pro-inflammatory immune cells (11, 54, 55). Our group recently reported that Ly6C^{Hi} monocytes/macrophages, which have been shown to promote inflammation, normally transition to Ly6C^{Lo}, which are more regenerative;

however, in diabetic wounds, a second wave of Ly6C^{Hi} macrophages are recruited during the reparative phase and fail to transition to the anti-inflammatory Ly6C^{Lo} cells contributing to a sustained pro-inflammatory environment (34). Furthermore, it has been shown that hyperglycemia and formation of AGEs impede the phagocytic capacity of macrophages ability to clear apoptotic neutrophils thereby promoting a sustained pro-inflammatory state (56, 57). Since the transition from a pro-to anti-inflammatory state is partly stimulated by neutrophil clearance, this results in a higher number of pro-inflammatory macrophages present in the wound. Additionally, studies have shown that monocyte/macrophage infiltration is sustained in diabetic mouse models due to alterations in expression of P-selectin and macrophage chemoattractant protein 1 (MCP-1) (54).

Endothelial cells

Endothelial cells line the luminal surface of blood vessels and are responsible for regulating vaso-constriction and -dilation by expression of vasoactive factors such as endothelial nitric oxide synthase (eNOS) (58). Reduced eNOS expression is associated with peripheral neuropathy and peripheral artery disease and contributes to decreased peripheral blood flow which, in turn, slows wound healing (59). Additionally, endothelial progenitor cells (EPCs) are required for neovascularization of the wound and eNOS stimulates mobilization of EPCs from the bone marrow (45, 60); thus the observed decrease in eNOS also contributes to impaired local angiogenesis during diabetes.

Keratinocytes and Fibroblasts

The late phase of wound healing is carried out by keratinocytes and fibroblasts. In non-healing diabetic wounds, a variety of alterations in keratinocyte function contribute to diminished epithelialization including defective keratinocyte migration and proliferation (61, 62), gap junction abnormalities (63), chronic inflammation and infection (54, 64), reduced angiogenesis (45, 60), oxidative stress (64), and abnormal expression of MMPs (23, 65, 66). The mechanisms associated with these processes have been reviewed elsewhere and we will not list them here (67–69).

Alterations in fibroblast function also contribute to dysfunctional epithelialization and delayed healing in diabetic wounds. These are primarily involved with decreased proliferation, increased apoptosis, and impaired migration to the wound site (20, 21, 70). Alterations in both keratinocytes and fibroblasts can be induced directly by hyperglycemia and AGE formation (18, 19, 22, 71, 72). Moreover, crosstalk between the two cell types is important for re-epithelialization and is dependent on a delicate balance between pro-inflammatory and anti-inflammatory cytokine expression in the wound (73).

Epigenetics and the wound healing response

Despite sharing a common genome, different cell types exhibit specific gene expression profiles that define their function. This is accomplished through epigenetic regulation, which modifies chromatin to activate or silence genes (74). Here, epigenetics is defined as heritable changes in transcription that are not caused by changes in the genetic code. Thus, these

modifications are not determined by mutation or permanent changes in nucleotide sequence but are maintained over time and passed through multiple generations of cell division.

There are three main types of epigenetic gene regulation: DNA modification, biochemical modification of histone tails, and ATP-dependent chromatin remodeling (75). These mechanisms are highly interdependent and work in concert with each other to either shut down or promote transcription of specific genes. During normal development, genes are regularly silenced and activated in a cell and/or tissue-specific manner. However, under disease conditions, the epigenetic machinery may often become dysregulated with dire consequences (75). For example, diabetes has been shown to induce changes in the epigenetic machinery that contribute to many of the associated complications. Important in the context of wound healing, epigenetic modifications have been shown to regulate downstream immune mediator expression in monocyte-derived macrophages and other immune cells (76–79). For example, several reports indicate that site-specific histone methylation has a role in macrophage polarization. Macrophage-specific knockout of mixed lineage leukemia 1 (*Mll1*) associates with reduced pro-inflammatory gene expression while the gene absent small and homeotic disks protein 1-like (*Ash1l*) promotes an anti-inflammatory phenotype by suppressing IL6 and TNF α production (76, 79). Additionally, the H3K27 demethylase, Jumonji domain-containing protein 3 (JMJD3) has been revealed to have roles in activation of both pro- and anti-inflammatory macrophage phenotypes. In murine macrophages, *Jmjd3* can be upregulated by both LPS and IL-4 and drive expression of pro-inflammatory genes or IL-4 target genes, respectively (77). Inhibition of JMJD3 and another H3K27 demethylase, UTX, reduced LPS-induced pro-inflammatory cytokine production in human primary macrophages (80) while induction of IRF4-dependent anti-inflammatory macrophage differentiation was reduced in *Jmjd3* knockout mice (81). Thus, JMJD3 may regulate macrophage polarization differently depending on the environmental/tissue-specific context.

Neutrophil activity has also been shown to be epigenetically regulated. Zimmerman *et al.* reported that purified human neutrophils maintain the IL-6 promoter in an inactive conformation but promote IL-6 expression after stimulation with the TLR8 agonist R848, corresponding with increased H3K4me3, H3K27ac, and H4ac, all marks of active transcription, at the IL-6 promoter (82). Further, neutrophils from systemic lupus erythematosus (SLE) patients have been reported to have reduced DNA methylation at interferon-regulated genes, suggestive of a role for DNA methylation in regulating the interferon gene program (83).

In addition to immune cells, the functions of other cells involved in wound healing have been shown to be epigenetically regulated (84). For example, JMJD3 is necessary for normal keratinocyte differentiation by promoting expression of differentiation-associated genes (85), while the H4K20 methyltransferase, SETD8, promotes normal keratinocyte proliferation and differentiation (86). Taken together, these reports suggest that epigenetic regulators could prove to be viable therapeutic targets for treating wound healing in diabetes and other conditions.

Epigenetic mechanisms and alterations in the diabetic wound

In the following sections, we will describe the fundamental mechanisms for each of type of epigenetic regulation and outline work showing how these processes are disrupted in diabetic complications and wound healing.

DNA modifications

DNA methylation—There are two different mechanisms by which DNA can be directly modified to modulate gene transcription: DNA methylation and DNA-hydroxy-methylation. DNA methylation is predominantly associated with transcriptional repression and is characterized by transfer of a methyl group to the cytosine ring of DNA by DNA methyltransferases (DNMTs) to form 5-methyl-cytosine (5mC) (87). DNMT3A and DNMT3B deposit *de novo* methylation marks while DNMT1 is responsible for maintaining these marks since these marks must be re-established with each cell division (88–90). In mammals, the vast majority of DNA methylation in somatic cells occurs at clusters of CpG dinucleotides termed CpG islands, and approximately 40% of genes contain these islands in their promoters (91). Methylation of CpG islands within the promoter can directly silence transcription by impeding transcription factor binding and also interacts with other factors, e.g., histone modifying enzymes, to inhibit transcription (92).

Several studies have shown a role for DNA methylation in diabetic wounds. A report by Shi and colleagues showed that DNMT1 inhibition by 5-aza-cytidine (5-aza-C) promoted M2-like macrophage formation and suppressed inflammation in bone marrow derived macrophages (BMDMs) (15). Interestingly, these mice were also protected against obesity-induced inflammation and insulin-resistance (15). Another study, using both genetic (*db/db*) and diet-induced obesity (DIO) mouse models of T2D, showed that DNMT1 was elevated in BMDMs and promoted a pro-inflammatory macrophage phenotype (16). Moreover, in this study, DNMT1 knockdown improved wound healing in *db/db* mice (16). The authors further show that 5-aza-C treatment promoted M2 polarization *in vitro*. Although the improvement in wound healing could be secondary to systemic effects due to improved obesity and insulin sensitivity, these data suggest that targeting DNMT1 in diabetic macrophages may be a viable therapeutic approach and warrants further investigation. Similar to macrophages, neutrophils in the non-healing wound are characterized by persistent recruitment and activation (51–53). Although reduced DNA methylation has been identified in neutrophils in other inflammatory diseases (83) there is currently insufficient data regarding DNA methylation status of neutrophils in diabetic wound healing.

Although active DNA methylation is known to be important for maintaining proper cell function in renewing epidermal tissue (93), less is known about its role in epithelialization during normal wound healing. Aberrant DNA methylation changes have been demonstrated in human diabetic foot ulcer fibroblasts (24). Surprisingly, instead of increased DNA methylation seen in previous studies, the authors report decreased DNA methylation compared to controls. Pathways affected by the differentially methylated genes were associated highly with wound healing, angiogenesis, and ECM assembly (24). In keratinocytes, the *MMP9* promoter is hypomethylated as a result of accumulation of diabetes-dependent AGE-conjugated bovine serum albumin (AGE-BSA) (22, 23, 94). This

suggests a potential role for active de-methylation regulating keratinocyte function during diabetic wound healing (see below).

In addition to its role in wound healing, several studies have demonstrated global changes in DNA methylation patterns associated with other diabetic complications. It is believed that DNA methylation plays a key role in maintaining “metabolic memory”, the concept that intensively controlling glucose levels for a number of years can have a long-term effect (24, 95). Importantly, changes in DNA methylation patterns have been found associated with insulin resistance and dysfunctional pancreatic β -cells from diabetic donors (96–99). Volkmar *et al.* showed that gene promoters were largely hypomethylated in diabetic pancreatic islets and functional analysis showed that subsets of differentially methylated and expressed genes were associated with pathways important for β -cell function (96). Taken together, the role of DNA methylation in diabetes appears to be complex and tissue-specific. Further studies will be necessary to determine how DNA methylation may be targeted in diabetes and, particularly, in wound healing.

DNA hydroxy-methylation—DNA methylation was originally thought to be irreversible and only occurred in the absence of DNMT1 during replication. Recently, however, an active process for DNA demethylation has been discovered whereby 5mC is sequentially oxidized by the Ten-Eleven Translocation (TET) family of enzymes to form 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and finally 5-carboxylcytosine (5caC), which is ultimately removed by base excision repair machinery (100). The 5hmC, 5fC, and 5caC moieties were originally thought to be intermediates in DNA de-methylation to restore gene transcription. However, studies have shown that 5hmC is stably deposited in gene bodies, promoters, and transcription factor binding site across the genome, suggesting that it may have an independent role in controlling gene transcription (100). 5fC and 5caC have also been seen to be stably distributed across the genome albeit at much lower levels (100).

There is evidence showing that DNA de-methylation plays an active role in diabetic wound healing. Yan and colleagues have demonstrated that AGE-BSA, which commonly accumulates as a result of hyperglycemia, retards keratinocyte mobility and proliferation by promoting *TET* expression which subsequently de-methylates the *MMP9* promoter (19, 22, 65, 94, 101). TNF α promotes *MMP9* expression in keratinocyte cell lines and is dependent on site-specific de-methylation of the *MMP9* promoter (23). This suggests that targeting DNA demethylation machinery may be a promising strategy for treating non-healing diabetic wounds. However, compared to DNA methylation, much less is known regarding the role of DNA demethylation in diabetic wound healing and further study is warranted.

Histone modifications

In eukaryotes, DNA is packaged into repeating units called nucleosomes by wrapping around multimeric histone proteins (102, 103). These nucleosomes are then further arranged in higher order structures to create chromatin fibers (104). In addition to efficiently packaging DNA, higher order chromatin structure acts to regulate gene transcription. When nucleosomes are organized into tightly packed bundles (heterochromatin), transcription is inhibited by barring access of transcriptional machinery (105–107). Conversely, when

chromatin is relaxed (euchromatin), the nucleosomes resemble beads on a string and this state is associated with active transcription (107).

Each histone protein is an octamer comprised of 2 sets of H2A, H2B, H3, and H4 proteins with a single histone H1 linker protein between nucleosomes (103). Each histone subunit has an N-terminal “tail” that protrudes away from the surface of the histone octamer creating an exposed surface (108). Here, histone modifying enzymes can methylate, acetylate, phosphorylate, or ubiquitylate specific residues on the histone tail (106). Depending on the modification and the specific residue modified, transcription can be either activated or silenced.

Histone lysine methylation/de-methylation—One highly studied histone modification is methylation. Unlike DNA methylation, which is always associated with transcriptional repression, histone methylation can either promote transcription or silence it depending on the target residue and the number of methyl groups added (106). For example, tri-methylation of lysine 4 on histone H3 (H3K4me3) (109) is a well-known activator of gene transcription while tri-methylation of lysine 27 on H3 (H3K27me3) is associated with transcriptional repression (110). Although H3K27me3 is linked with transcriptional silencing, H3K27 mono-methylation (H3K27me1) has been documented to promote transcription (110). Importantly, both of these marks are involved in macrophage polarization as alternatively activated macrophages have been found to have increased H3K4 methylation and decreased H3K27 methylation in promoters of anti-inflammatory genes (77). Here, an increased level of the H3K27 de-methylase *Jmjd3* was shown to be responsible for decreased di- and tri-methylation of H3K27 (77).

Histone H3K4 can be methylated by several different members of the SET domain-containing family of proteins. In particular, MLL1 has been shown to promote expression of inflammatory genes in a NFκB-dependent manner (66, 76, 111). Our group has shown that MLL1 is important for normal tissue repair by catalyzing H3K4me3 deposition at pro-inflammatory genes in macrophages during the inflammation phase of wound healing (14). We further showed that mice bearing a myeloid-specific MLL1 deletion had delayed wound healing and decreased pro-inflammatory cytokine production. Related to diabetic wound healing, our data showed that monocytes isolated from T2D patients exhibit increased *MLL1* expression implying that *MLL1* expression is dynamically regulated during the transition to the proliferation phase. To date, this is only study imparting MLL1 with a role in diabetic wound healing. Thus, more investigation is necessary to determine whether MLL1 may be a viable therapeutic target.

Our laboratory and others have shown that expression of *Jmjd3*, the histone demethylase targeting H3K27me3, is increased and H3K27me3 decreased in wound macrophages in a DIO mouse model of diabetes (11, 17). H3K27me3 is a transcriptionally repressive mark that promotes heterochromatin formation in the promoters of genes (112, 113); thus, JMJD3 acts to promote transcription. In a DIO model of diabetes, we found that JMJD3 promotes expression of IL-12 in wound macrophages, and this phenomenon can be reversed by JMJD3 inhibition (11). Similarly, a report by Natoli and colleagues showed that *Jmjd3* expression was increased in macrophages in response to inflammatory stimuli and was

responsible for regulating developmental genes expression during bone marrow differentiation (17). Studies have shown that IL-6 expression in normal neutrophils is epigenetically regulated in response to TLR activation and is associated with increased H3K4me3, and H3K27ac, and acetylated histone H4 (82). However, there is a lack of data regarding epigenetic regulation in neutrophils during diabetic wound healing warranting further study.

Other studies suggest *Jmjd3* expression is required for re-epithelization (114, 115). For example, JMJD3 has been shown to promote keratinocyte migration to the wound site by promoting Notch1 expression (114). Interestingly, Shaw and Martin demonstrated increased *Jmjd3* expression in epithelial cells at the leading edge of normal wounds with a concurrent decrease in *Ezh2*, the HMT responsible for depositing the H3K27 methylation mark (115). Levels of *Jmjd3* were upregulated in wounds at day 1 and abated over time. Thus, JMJD3 appears to be tightly regulated in both macrophages and keratinocytes, where higher expression is important early in wound healing but is down-regulated as the wound resolves; where this regulation appears to be absent in the diabetic wound. However, data concerning JMJD3 and EZH2 in keratinocytes has been limited to normal wound conditions. Further study is necessary to determine whether they play a role in the context of diabetic wounds.

Other HMTs have been ascribed with roles processes that could be involved in diabetic wound healing. ASH1L targets histone H3 and has been found to be associated with methylation of K4, K9, K20 and K36 at active gene promoters (116, 117). *Ash1l* deletion leads to delayed re-epithelialization in normal wounds (118). Despite this, no role for ASH1L has been described in diabetic wound healing. Additionally, another member of the SET domain family of HMTs, SET7, is responsible for mono-methylation of histone H3K4 (H3K4me1) (119). This mark is typically found in enhancer regions of genes where it poises genes for active transcription (120). It has been shown that hyperglycemia increases SET7 expression in vascular endothelial cells and drive expression of pro-inflammatory genes (119).

Taken together, it is clear that histone lysine methylation plays variable roles in wound healing depending on the cell type and the specific histone tail residue modified. Based on limited studies thus far, MLL1 and JMJD3 are promising potential therapeutic targets.

Histone arginine methylation/de-methylation—Histones can also be methylated at arginine residues. Here, arginine is mono- or dimethylated by protein arginine methyl transferases (PRMTs) (121). Arginine has two exposed terminal amino groups and di-methylation can occur either with 2 methyl groups on one amino group (asymmetric) or with both groups mono-methylated (symmetric) (121). Compared to lysine modification, there is much less information regarding histone arginine methylation in diabetic complications, including wound healing. A few studies have shown that PRMTs are involved in insulin secretion in β -cells and some are overexpressed in diabetes (122–124). However, PRMTs can, not only methylate histones, but also non-histone proteins (121). It is currently not clear whether arginine modifications play a histone-dependent or independent role (or both) in diabetic complications. There is evidence that PRMT1 and CARM1/PRMT4 form a complex with NF κ B and methylate H3 arginine residues to drive expression of NF κ B gene

targets (125, 126) suggesting that arginine methylation could be involved in promoting pro-inflammatory responses. However, studies involving histone arginine methylation in diabetes and, in particular, diabetic wound healing are minimal and warrant further investigation.

Histone acetylation/de-acetylation—Another well-characterized modification is histone acetylation. Histone acetylation is carried out by histone acetyl-transferases (HATs) which catalyze the transfer of an acetyl group from acetyl-coenzyme A (Ac-CoA) to the terminal amino group of lysine residues on the histone (127). Lysine's terminal amino group is positively charged under physiological conditions and interacts with the negatively charged phosphates in the DNA backbone to form a stable complex between the histone and DNA (108). Acetylation of this amino group neutralizes its charge thereby breaking the interaction with DNA and allowing the histone to be repositioned and allow access to transcriptional machinery (128). Thus, histone acetylation is distinct in that it is exclusively associated with transcriptional activation. This process can also be reversed with the removal of the acetyl group by histone deacetylases (HDACs) to shut down gene expression (129, 130).

Given that Ac-CoA is a major byproduct produced during fatty acid metabolism, it seems likely that histone acetylation would play a role in the development of diabetes and potentially be involved in regulating wound healing processes in the context of obesity-induced inflammation. Indeed, histone acetylation has been linked to cellular metabolism and it has been shown that high fat diet can affect acetyl-CoA and histone acetylation levels (131–134). The majority of these studies focus on cancer. However, there are a few reports regarding histone acetylation and diabetes and wound healing. Global decreases in H3K9 and H3K23 acetylation have been reported in diabetic mouse livers (135). In this study, acetylated H3K9 was shown to be decreased in the promoter of *Glut2*, a glucose transporter important for maintaining glucose homeostasis (136, 137), and could be reversed by treatment with the diabetic inhibitor exendin-4 (135). Related to this, a report showed that exendin-4 can protect against diabetic retinopathy by inducing acetylation of histone H3 at the *SOD3* promoter to drive its expression in endothelial cells (138). Further, reports from Spallotta *et al.* and Melchionna *et al.* showed that when treated with HDAC inhibitors, mice exhibited increased keratinocyte proliferation and improved wound healing (139, 140). These studies suggest that histone de-acetylation may be a promising therapeutic target. However, aside from these few studies, there is a paucity of information related to histone acetylation in diabetes and diabetic wound healing.

Other histone modifications—Histones can also be phosphorylated and ubiquitinated. These marks have established roles in transcriptional regulation and have been extensively reviewed elsewhere (141–143). However, no work has been done related to these marks in wound healing or diabetes and their roles, at present, remain undefined.

ATP-dependent chromatin remodeling

Aside from histone acetylation, interactions between histones and DNA can also be modulated by remodeling complexes fueled by ATP-hydrolysis. All ATP-dependent remodeling complexes contain an ATPase of the SNF2 family and have been classified into

two groups based on their subunits, the SWI2/SNF2 and ISWI groups (144). The activity of each group is similar in that they both utilize ATP. However, while SWI2/SNF2 can be activated by naked DNA as well as nucleosomes (145), ISWI complex must be in contact with nucleosomes containing histones with intact amino-terminal tails (146). The consequence of remodeling is dependent on the context of the nucleosomes at a given promoter and can result in either transcriptional activation or repression (144).

Although ATP-dependent remodeling has been extensively studied, the majority of work in this field has focused on DNA damage/repair in cancer and very little is known about these mechanisms in diabetes or wound healing. Some studies, however, have shown that forced increase of cellular ATP can accelerate wound healing by promoting neovascularization and collagen production (147). The increased ATP levels led to increases in SWI/SNF complex components BRG1 and BRM ATPases and promoted a novel pathway in anti-inflammatory macrophages characterized by increased pro-inflammatory cytokine MCP-1 chemokine production. Related to this, it has been shown that *Brg1* deletion impairs keratinocyte terminal differentiation (148) suggesting a more general mechanism for ATP-dependent remodeling.

Conclusions

Diabetic wounds are characterized by persistent inflammation secondary to alterations in immune cell function. Here, we have described epigenetic changes that alter immune cell phenotypes as well as other cells important for proper healing (fig. 1). As new mechanisms important for the regulation of healing are discovered, progress towards cell-specific targeted therapies necessary for the treatment of pathologic healing will accelerate.

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Abbreviations:

5caC	5'-carboxy-cytosine
5fC	5'-fluoro-cytosine
5hmC	5'-hydroxymethyl-cytosine
5mC	5'-methyl-cytosine
Ac-CoA	Acetyl Coenzyme A
AGE	Advanced Glycation End-product

AGE-BSA	Advanced Glycation End-product – Bovine Serum Albumin
ASH1L	Absent Small and Homeotic Disks Protein 1-like
BMDM	Bone Marrow-Derived Macrophage
DAMP	Damage-Associated Molecular Pattern
DIO	Diet-induced Obesity
DNMT	DNA Methyltransferase
ECM	Extracellular Matrix
eNOS	Endothelial Nitric Oxide Synthase
H3K4	Histone H3 Lysine 4
H3K27	Histone H3 Lysine 27
HAT	Histone Acetyltransferase
HDAC	Histone De-acetylase
HMT	Histone Methyltransferase
JMJD3	Jumonji Domain-containing Protein 3
LPS	Lipopolysaccharide
MLL1	Mixed Lineage Leukemia 1
MCP-1	Macrophage Chemoattractant Protein 1
MMP	Matrix Metalloprotease
NET	Neutrophil Extracellular Trap
NO	Nitric Oxide
PAD4	Peptidyl Iminase 4
PAI-1	Plasminogen Activator Inhibitor 1
PMN	Polymorphonuclear Neutrophils
SLE	Systemic Lupus Erythematosus
T2D	Type 2 Diabetes
TET	Ten Eleven Translocase

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Figure 1:
Overview of epigenetic mechanisms regulating cells involved in wound healing.