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Epigenetic changes caused by diabetes and their potential role in the development of periodontitis

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ABSTRACT

Aims/Introduction: Periodontal disease, a chronic inflammation induced by bacteria, is closely linked with diabetes mellitus. Many complications associated with diabetes are related to epigenetic changes. However, the exact epigenetic changes whereby diabetes affects periodontal disease remain largely unknown. Thus, we sought to investigate the role of diabetes-dependent epigenetic changes of gingival tissue in the susceptibility to periodontal disease.

Materials and Methods: We studied the effect of streptozotocin-induced diabetes in minipigs on gingival morphological and epigenetic tissue changes. Accordingly, we randomly divided six minipigs into two groups: streptozotocin-induced diabetes group, $n = 3$; and non-diabetes healthy control group, $n = 3$. After 85 days, all animals were killed, and gingival tissue was collected for histology, deoxyribonucleic acid methylation analysis and immunohistochemistry.

Results: A diabetes mellitus model was successfully created, as evidenced by significantly increased blood glucose levels, reduction of pancreatic insulin-producing β-cells and histopathological changes in the kidneys. The gingival tissues in the diabetes group presented acanthosis of both gingival squamous epithelium and sulcular/junctional epithelium, and a significant reduction in the number and length of rete pegs. Deoxyribonucleic acid methylation analysis showed a total of 1,163 affected genes, of which 599 and 564 were significantly hypermethylated and hypomethylated, respectively. Immunohistochemistry staining showed that the hypomethylated genes – tumor necrosis factor-α and interleukin-6 – were positively expressed under the junctional epithelium area in the diabetes group.

Conclusions: Diabetes mellitus induces morphological and epigenetic changes in periodontal tissue, which might contribute to the increased susceptibility of periodontal diseases in patients with diabetes.

INTRODUCTION

Diabetes mellitus represents a group of metabolic disorders characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both[1]. According to the latest International Diabetes Federation data, one in every 11 adults worldwide had diabetes in 2019. Diabetes mellitus can cause many complications, such as retinopathy, nephropathy, peripheral neuropathy, vascular lesions and impaired wound healing[1,2]. One such complication is periodontitis, a common chronic inflammatory disease caused by bacterial biofilm (dental plaque) colonization of the teeth surface that results in irreversible tissue damage[3]. Susceptibility to the disease is also dependent on factors other than the absolute level of dental plaque, such as genetic polymorphisms, environmental risk

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factors, lifestyle and epigenetic changes[4–6]. Diabetes mellitus is a major environmental risk factor for periodontal disease, increasing the prevalence, extent and severity of periodontitis[3,7–9]. Importantly, the relationship between these two complex and chronic diseases is bidirectional[3,10–12]. Diabetes mellitus is associated with increased expression of inflammatory-related markers such as prostaglandin E2, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6, as well as defects in polymorphonuclear leucocyte activity, which might induce or accelerate the development of periodontitis[10,13]. In contrast, severe periodontitis is also associated with poorer glycemic control[3,11] and significantly higher glycated hemoglobin A1c[14]. Despite the extensive research carried out over the past years, the exact mechanisms linking these two complex diseases are not entirely understood, thus deserving further investigation.

Epigenetics is defined as the study of heritable and biological changes in gene function that are independent of deoxyribonucleic acid (DNA) sequence[15]. One of the most important epigenetic changes is DNA methylation, which mainly occurs at CpG sites where a cytosine nucleotide is followed by a guanine nucleotide from the 5′ → 3′ direction. Such modification is involved in many biological processes, especially in oncogenesis and inflammation[16–18].

The pathogenesis of diabetes mellitus involves both genetic and environmental factors[19,20]. Life-long environmental exposure to nutritional, endocrine and chemical perturbation might change gene expression through epigenetic modifications, such as DNA methylation, histone modification and microribonucleic acids, thus affecting cell phenotypic differentiation[21,22]. Importantly, many of the complications during diabetes are associated with epigenetic modifications[21,23,24]. However, the exact diabetes-associated pathogenetic changes implicated in the susceptibility to periodontitis remain largely unknown. Thus, we sought to understand the epigenetic changes that occur in periodontal tissue during diabetes. To that end, we investigated morphological and epigenetic DNA methylation changes of gingival tissues using a diabetic minipig model. In addition, we discuss the relationship between these changes and the susceptibility of periodontal tissues to inflammation, thus providing a better understanding of the pathophysiology and therapeutic management of periodontitis.

METHODS

Animals

The project was fully compliant with the Guide for Care and Use of Laboratory Animals (8th edition, released by the National Research Council, Washington DC, USA). All animal procedures were approved by the Nanjing University Animal Ethics Committee, and the experiments were carried out in biosafety level 3 (BSL-3) facilities at the Department of Comparative Medicine at the General Hospital of Eastern Theater Command of PLA (Nanjing, China 210002).

A total of six 1-year-old Guangxi Bana minipigs, weighing 60–80 kg, were used in the study. All animals were given clean ordinary pig feed and water ad libitum. The room temperature was 16–18°C. The animals were randomly divided into two groups: diabetes mellitus group (n = 3) and non-diabetes group (n = 3).

Induction of diabetes

The animals were anesthetized with ketamine (Hospira, Lake Forest, IL, USA)/xylazine (Xyla-Ject; Phoenix, St. Josephs, MO, USA) via intramuscular injection (20 mg/kg) and were weighed afterwards. Blood samples were taken from the superior vena cava of each animal before the induction of experimental diabetes, and blood glucose levels were measured as a baseline at the Stomatological Hospital affiliated to Nanjing University, Nanjing, China. Three pigs were administered with high-dose streptozotocin (STZ; 150 mg/kg, Sigma, St Louis, MO, USA) diluted in 9.5 mL/mg sterile saline (0.9% NaCl injection, USP; Baxter, Deerfield, IL, USA) via the auricular vein. Blood samples were obtained at days 1, 45, 65 and 85 after the injection. All pigs were killed after 85 days using a pentobarbital overdose. Then, the gingival, kidney and pancreatic tissues of each group were collected. A fraction of the fresh gingival tissue was fixed with TRIzol (Invitrogen, Carlsbad, CA, USA) for epigenetic testing, and the other was fixed with 4% paraformaldehyde for histology and immunohistochemistry analysis.

Morphological evaluation

Samples fixed in 4% paraformaldehyde were dehydrated through graded ethanol baths (100–70%), cleared in xylene and then embedded in paraffin wax. The paraffin blocks were serially sectioned at 5 μm thickness, using a Leica RM2125 rotary microtome (RM2125 RTS; Leica, Wetzlar, Germany). The sections were stained with hematoxylin–eosin, scanned using the Pannoramic 250 FLASH scanner and evaluated using the Pannoramic viewer 1.15.2 software (3D HISTECH, Budapest, Hungary). The length and density of rete pegs were analyzed in five random areas per slice using Image J software (National Institutes of Health, Bethesda, MA, USA).

DNA methylation level in the gingival tissues of diabetic and non-diabetic minipigs

Total DNA was extracted and purified using Qiagen DNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocols. The quality of purified DNA was verified with agar gel (1.0%) electrophoresis and the DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Methylated DNA immunoprecipitation sequencing (MeDIP-seq) library was prepared as described previously with minor modifications[25].

For MeDIP-seq, extracted genomic DNA from gingival tissue was sonicated to ~200–500 bp with a bioruptor sonicator (Diagenode, Seraing, Belgium). Approximately 1 μg of sonicated DNA was end-repaired, A-tailed and ligated to single-end adapters following the standard Illumina Genomic DNA

Sample Prep Kit protocol. The unligated adapters were eliminated using AMPure XP beads (Beckman-Coulter, Brea, CA, USA), after which the adaptor-ligated DNA was used for immunoprecipitation using a mouse monoclonal anti-5-methylcytosine antibody (Diagenode, Liege, Belgium). For this, DNA was heat denatured at 94°C for 10 min, rapidly cooled on ice and immunoprecipitated with 1 μL primary antibody overnight at 4°C with rocking agitation in 400 μL immunoprecipitation buffer (0.5% bovine serum albumin in phosphate-buffered saline [PBS]). To recover the immunoprecipitated DNA fragments, 100 μL of protein G magnetic beads (Life Technologies, Carlsbad, CA, USA) were added and incubated for an additional 2 h at 4°C with agitation. Subsequently, five immunoprecipitation washes were carried out with ice-cold immunoprecipitation buffer. Non-specific mouse immunoglobulin G immunoprecipitation was carried out in parallel to MeDIP as a negative control. Washed beads were resuspended in TE buffer with 0.25% sodium dodecyl sulfate and 0.25 mg/mL proteinase K for 2 h at 65°C, and then allowed to cool down to room temperature. MeDIP and supernatant DNA were purified using Qiagen MinElute columns and eluted in 16 μL elution buffer (Qiagen). A total of 14 cycles of polymerase chain reaction were carried out on 5 μL of the immunoprecipitated DNA using single-end Illumina polymerase chain reaction primers (Illumina, San Diego, CA, USA). The resulting reactions were purified with Qiagen MinElute columns, after which a final size selection (~300–600 bp) was carried out using AMPure XP beads. Libraries were quality controlled with (Agilent 2100 Bioanalyzer, Santa Clara, CA, USA). An aliquot of each library was diluted in elution buffer to 5 ng/μL, and 1 μL was used in real-time polymerase chain reaction reactions to confirm the enrichment in methylated regions.

Sequencing

The library was denatured with 0.1 mol/L NaOH to generate single-stranded DNA molecules, loaded onto channels of the flow cell at 8pmol/L concentration and amplified *in situ* using HiSeq 3000/4000 PE Cluster Kit (#PE-410-1001; Illumina). Sequencing was carried out by running 2 × 150 cycles on Illumina HiSeq 4000 according to the manufacturer's instructions.

Data analysis

Image analysis and base calling were carried out using Off-Line Basecaller software (OLB V1.8, Illumina, SanDiego, CA, USA). After Solexa CHASTITY quality filter, clean reads were aligned to the *Sus scrofa* genome (UCSC version susScr3) using Bowtie2 software (V2.2.7, http://bowtie-bio.sourceforge.net/bowtie2/index.shtml). MeDIP-enriched regions (peaks) with statistical significance were identified for each sample, using a *q*-value threshold of 10-5 by MACS v2. The messenger ribonucleic acid-associated MeDIP-enriched regions (peaks) were annotated by the nearest gene using the newest UCSC RefSeq database. Statistically significantly differentially methylated regions within promoters between the two groups were identified by diffReps (cut-off log2FC = 1.0, *P*-value = 1.0). The messenger ribonucleic acid-associated differentially methylated regions within promoters were annotated by the nearest gene using the UCSC RefSeq and database of multiple databases integration.

Genome-wide DNA methylation analysis was carried out in both groups. For the genetic function and pathway identification, Gene Ontology (GO) term (http://www.geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg) enrichment analyses were carried out.

Immunohistochemistry

The paraffin-embedded gingival tissue blocks were sectioned to a thickness of 4 μm and subjected to immunohistochemistry for changes in blood vessels, TNF-α and IL-6. Immunohistochemical staining was carried out according to a previously reported protocol with some modifications[26]. Briefly, sections were deparaffinized/rehydrated in a xylene–ethanol–PBS serial procedure. Then, antigen retrieval was carried out using citrate (pH 6.0) in a microwave oven followed by blocking of endogenous peroxidase with 3% H_2O_2 to reduce non-specific background staining. Then, the slices were washed three times in PBS on a rocking device and subsequently pretreated with 3% bovine serum albumin for 30 min at room temperature. Next, samples were incubated with PBS-diluted primary antibodies against α-smooth muscle actin (1:500; Abcam, no. ab7817, Cambridge, MA, USA), TNF-α (1:100; Abcam, no. ab6671), IL-6 (1:500; Abcam, no. ab6672) at 4°C overnight. Diluted horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:200; Servicebio, Wuhan, China) was incubated for 50 min at room temperature followed by incubation with 3,3′-diaminobenzidine (Servicebio) as chromogen. Slides were routinely mounted and scanned using the Pannoramic 250 FLASH scanner, and evaluated using the Pannoramic viewer 1.15.2 software (3D HISTECH). Image J software (National Institutes of Health) was used to count blood vessels and positive stained cells in three different ×100 fields for each sample.

Statistical analysis

The results were analyzed using SPSS 21.0 statistical software (IBM Corp., Armonk, NY, USA). Data are expressed as the mean ± standard deviation. Blood glucose level comparison between the two groups was carried out using a two-way ANOVA method, and two-tailed Student's unpaired *t*-test was used to assess differences in the morphological histology changes. $P < 0.05$ was considered statistically significant.

RESULTS

Diabetes induction

Before STZ induction, the mean fasting blood glucose level of all animals was 4.43 ± 1.26 mmol/L (mean ± standard error of the mean). The mean fasting blood glucose level in the diabetes mellitus group was significantly increased 45 days after STZ

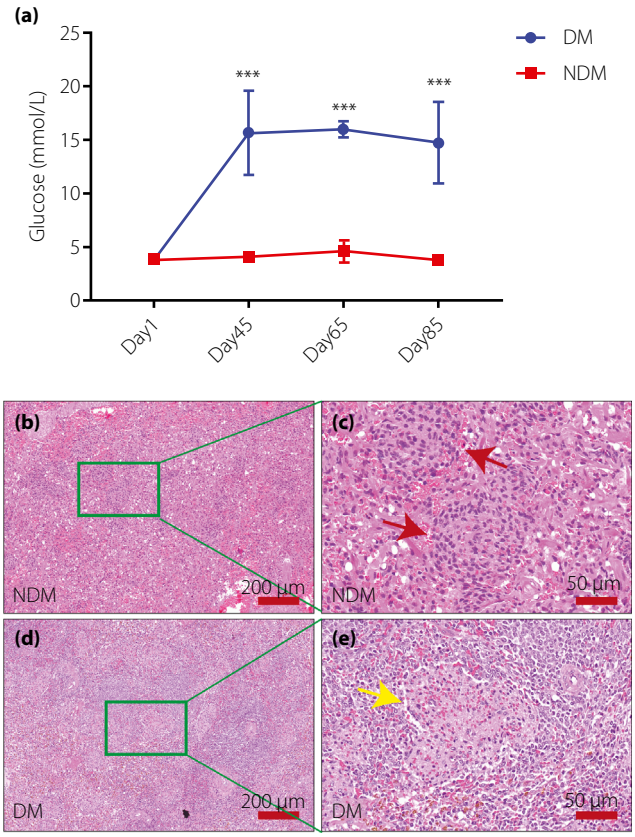

Figure 1 | (a) Fasting blood glucose levels and (b–e) representative images of pancreas (hematoxylin–eosin staining) from the diabetes (DM) and non-diabetes (NDM) groups. Compared with the non-diabetes group, the glucose level was significantly increased in the diabetes group from day 45 after intravenous infusion of streptozotocin (STZ). The islets of Langerhans in the diabetes group were atrophied and irregular compared with those in the non-diabetes group. The β-cells in the islets of the (e) diabetes group (yellow arrow) were reduced and severely degranulated compared with those in the (c) non-diabetes group (red arrows).

administration compared with the non-diabetes mellitus group and remained elevated throughout the observational period (Figure 1a).

Histological analysis

STZ administration induced structural changes in pancreatic, renal and gingival tissues of the diabetes mellitus group. Although the non-diabetes mellitus group showed clear and regular structures, the pancreatic islets were significantly reduced in diabetic pigs, showing atrophied and irregular shapes. Pancreatic β-cells were severely degranulated and unevenly arranged (Figure 1b,c vs d,e). STZ-induced hyperglycemia also resulted in damaged renal tubules with a reduction in their number. Most tubules showed signs of degeneration, presenting vacuolization containing prominent cytoplasmic vacuoles and pyknotic nuclei (Figure S1). STZ injection induced gingival epithelium morphological changes. The squamous epithelium of the diabetes mellitus group (Figure 2f,g) showed acanthosis and shortened irregular rete pegs compared with the non-diabetes mellitus group (Figure 2b,c). In addition, the subcutaneous connective tissue in the diabetes mellitus group also became looser and thinner compared with the non-diabetes mellitus group. Diabetes mellitus significantly reduced the number and length of rete pegs compared with the non-diabetes mellitus group (Figure 2d,h). The gingival sulcular/junctional epithelium of the diabetes mellitus group was also affected by the injection, with the spinosum layer of the junctional epithelium showing hyperplasia (Figure 2a,e). The immunohistochemical staining showed a significant decrease in gingival tissue blood vessel numbers in the diabetes mellitus group (Figure 2i–k). The number of cells positive for TNF-α and IL-6 in the diabetes mellitus group was significantly higher than those in the non-diabetes mellitus group, respectively ($P < 0.05$).

DNA methylation analysis

Genome-wide DNA methylation analysis showed that STZ-induced diabetic status altered the methylated expression of 1,163 genes, of which 599 and 564 genes were significantly hyper- and hypomethylated, respectively, in the diabetes mellitus versus the non-diabetes mellitus group (Figure 3a).

To further identify the genetic functions and pathways possibly affected by the methylation changes in the gingival tissue of the diabetes mellitus group, we used GO term and KEGG pathway enrichment analyses. A total of 679 functional gene categories were found in the diabetes group, of which 290 and 389 were hyper- and hypomethylated, respectively. Genes responsible for biological processes comprised the largest portion (534 of 679, hyper 231 vs hypo 312), then, cellular components (69 of 679, hyper 25 vs hypo 44) and molecular functions (67 of 679, hyper 34 vs hypo 33). The top 20 GO terms of differentially expressed genes in biological processes (BP) showed that hypermethylated genes were involved in smooth muscle contraction, lipid transport, hormone secretion, blood vessel size regulation and immune process, whereas hypomethylated genes were involved in lipid regulation, inflammation response, mononuclear cell migration, monocyte chemotaxis, regulation of tissue remodeling and response to external stimuli. (Figure 3b,c). In addition, some differentially methylated GO categories associated with other important signaling pathways possibly related to morphological changes and periodontal tissue status were enriched in the diabetes mellitus group. In the GO hypermethylated BP, these included glucose metabolism, T-cell apoptotic process, regulation of cell differentiation, complement activation, stem cell proliferation, regulation of cell fate commitment, lymphocyte apoptotic process, cellular response to chemical stimulus, insulin-like growth factor receptor signaling pathway and regulation of cell maturation; whereas in hypomethylated BP, they included neutrophil chemotaxis, cell

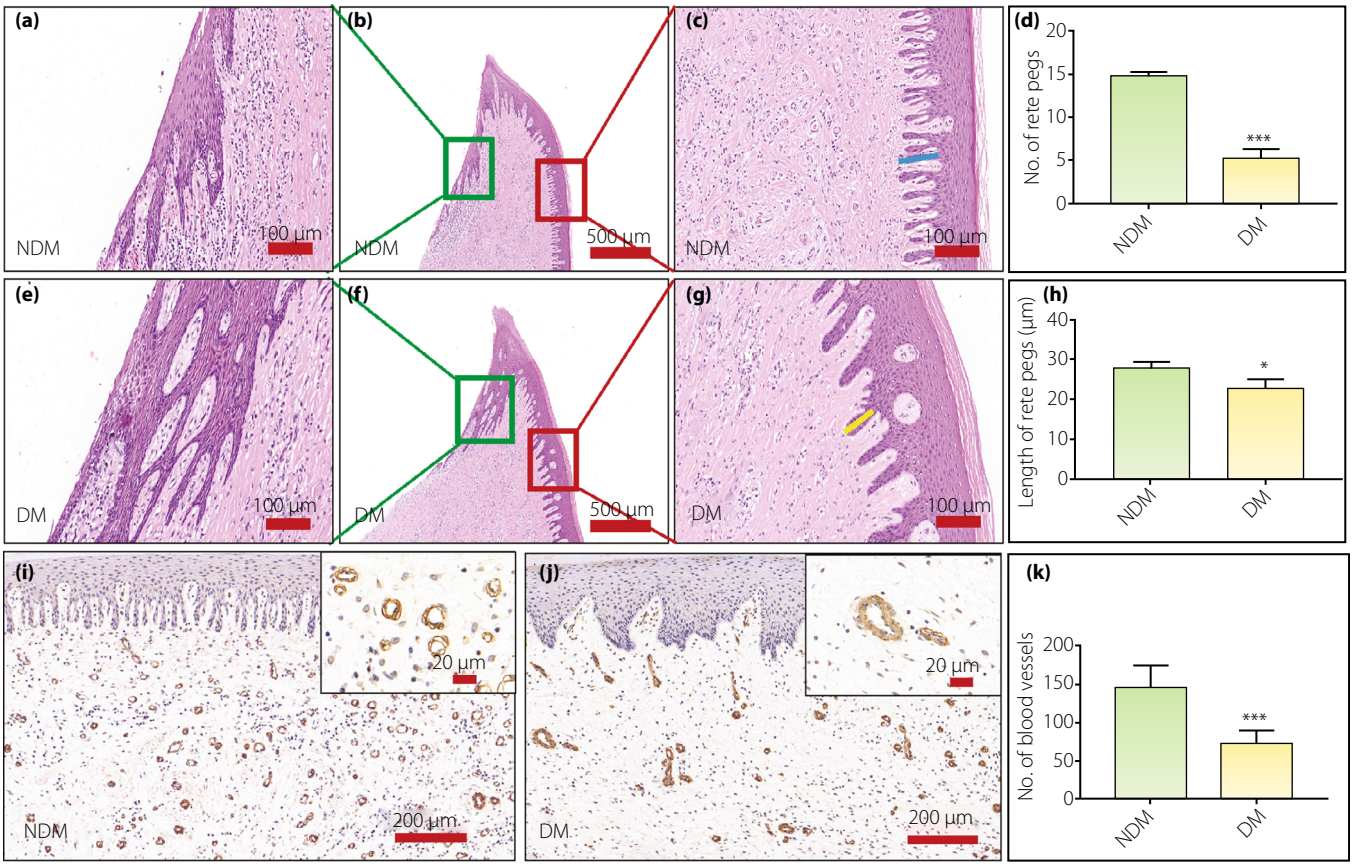

Figure 2 | Histological (hematoxylin–eosin staining) and immunohistochemical examination of gingival tissue. Compared with the (e–g) non-diabetes (NDM) group, the gingival epithelium of the (a–c) diabetes (DM) group was affected by the streptozotocin injection, showing acanthosis and shortened irregular rete pegs (dermal papillae) in the squamous epithelium (b, c vs f, g). The (c) blue line and (g) yellow line represent the length of rete pegs in the non-diabetes and diabetes groups, respectively. The gingival connective tissue of the diabetes group became looser and thinner than that of the non-diabetes group. (d,h) The streptozotocin injection significantly reduced the number and length of the rete pegs in the diabetes group compared to the non-diabetes group ($P < 0.05$). Acanthosis of gingival sulcular/junctional epithelium was also observed in the (e) diabetes group compared with that of the (a) non-diabetes group. Immunohistochemical α-smooth muscle actin (α-SMA) staining of gingival blood vessels showed that, compared with the (i) non-diabetes group, (j) the total number of blood vessels in the diabetes group was (k) significantly decreased ($P < 0.05$).

growth, cell differentiation, cell adhesion, blood vessel development, T-cell regulation, lymphocyte aggregation, monocyte chemotaxis, detection of external stimulus, bone remodeling, toll-like receptor signaling pathway and mast cell activation involved in immune response. Tables S1 and S2 list the top 20 enrichment differentially expressed genes of both hyper- and hypomethylated BP. Besides the top 20 categories, Table S3 lists other interesting GO functional enrichment differentially expressed genes of both hyper- and hypomethylated BP possibly related to morphological changes and periodontal tissue status in the diabetes mellitus group.

KEGG pathway analysis showed that different signal pathways, such as cyclic adenosine monophosphate, 5′ adenosine monophosphate-activated protein kinase and phosphatidylinositol 3 kinase–protein kinase B, were involved in the hypermethylation process, whereas peroxisome proliferator-activated receptor, Janus kinase-signal transducer and activator of transcription, forkhead box O, TNF, human-inducible factor-1, vascular endothelial growth factor and nucleotide-binding oligomerization domain-like receptor signal pathways were involved in the hypomethylation process. Nuclear factor-kappa B was involved in both processes. The immune system was also significantly affected by STZ injection; however, only a small fraction of the signaling pathways were related to hypermethylation, such as complement and coagulation cascades and natural killer cell-mediated cytotoxicity, whereas most signaling pathways were related to hypomethylation, such as T-cell and B-cell receptor signaling; T helper type 1, T helper type 2 and T helper type 17 cell differentiation; Toll-like receptor; hematopoietic cell lineage; intestinal

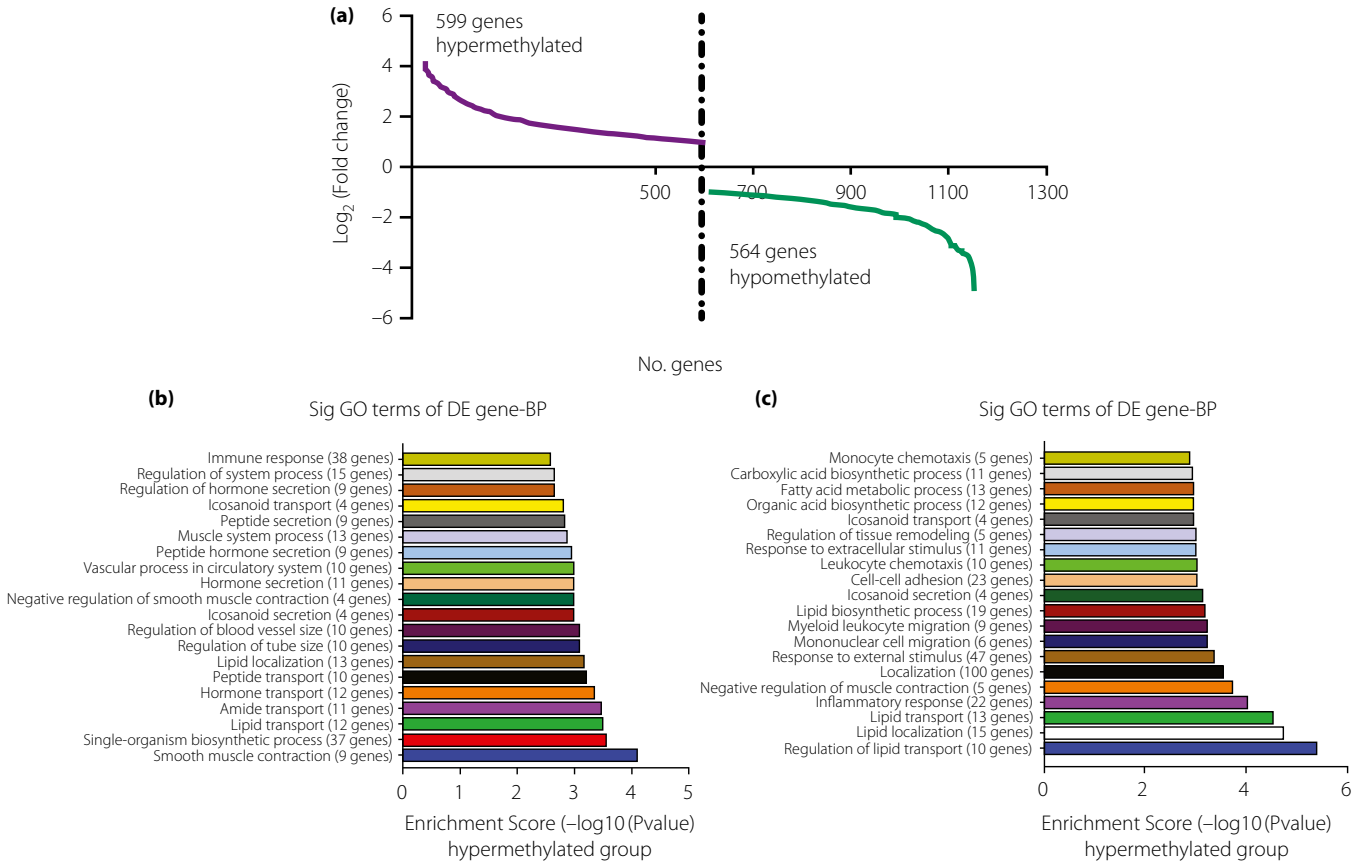

Figure 3 | Methylated deoxyribonucleic acid immunoprecipitation sequencing (MeDIP-seq) analysis and the further Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of the gingival tissues. (a) In total, 1,163 genes in the diabetes group were methylated compared with the non-diabetes group, of which 599 were hypermethylated and 564 were hypomethylated. (b,c) Separately listed the top 20 biological processes (BP) of the differentially expressed genes, including both hyper- and hypomethylated categories. The hypermethylated BP included smooth muscle contraction, single-organism biosynthetic process, lipid transport, regulation of hormone secretion, immune response and so on, whereas hypomethylated BP included regulation of lipid transport, inflammatory response, regulation of tissue remodeling, response to external stimulus, cell-to-cell adhesion, leucocyte chemotaxis, monocyte chemotaxis and so on.

immune network for immunoglobulin A production; and nucleotide-binding oligomerization domain-like receptor signaling. Furthermore, metabolic and endocrine systems were also affected by STZ injection. All of these processes could contribute to the morphology and structural changes of the gingival tissue (Tables S4,S5).

As inflammation regulating cytokines are key factors affecting periodontal tissue status, genes belonging to such systems and related to periodontal disease were selected from GO and KEGG analysis. Hypermethylated genes included complement component 1q (*C1QS*), mannan-binding lectin-associated serine protease-1 (*MASP1*), C-C chemokine ligand-5 (*CCL5*), complement component 4 binding protein (*C4BPA*), interferon-β (*INFB*) and transforming growth factor beta (*TGFB*); whereas hypomethylated genes included CXC chemokine ligands 9, 10, 12 (*CXCL9*, *CXCL10*, *CXCL12*), *CD86*, complement C5a receptor 1 (*C5AR1*), complement component 3 (*C3*), TNF-related genes (*TNFr*), IL-6 (*IL6*) and C-C motif chemokine ligand 22 (*CCL22*). To further investigate the protein expression of such genes in gingival tissue, TNF-α and IL-6 were selected for immunohistochemistry staining observation. The results showed that both genes were positively expressed and significantly increased under the junctional epithelium area in the diabetes mellitus versus the non-diabetes mellitus group (Figure 4).

DISCUSSION

The pathogenesis of both diabetes and periodontitis is influenced by genetic factors and epigenetic processes[27,28]. Patients with diabetes show increased prevalence and severity of periodontitis[29,30]. Although diabetes is associated with important epigenetic changes, the exact changes implicated in the susceptibility to periodontitis remain unknown.

In the present study, we investigated the morphological and epigenetic DNA methylation changes of gingival tissues using a diabetic minipig model. After STZ injection, a sustained

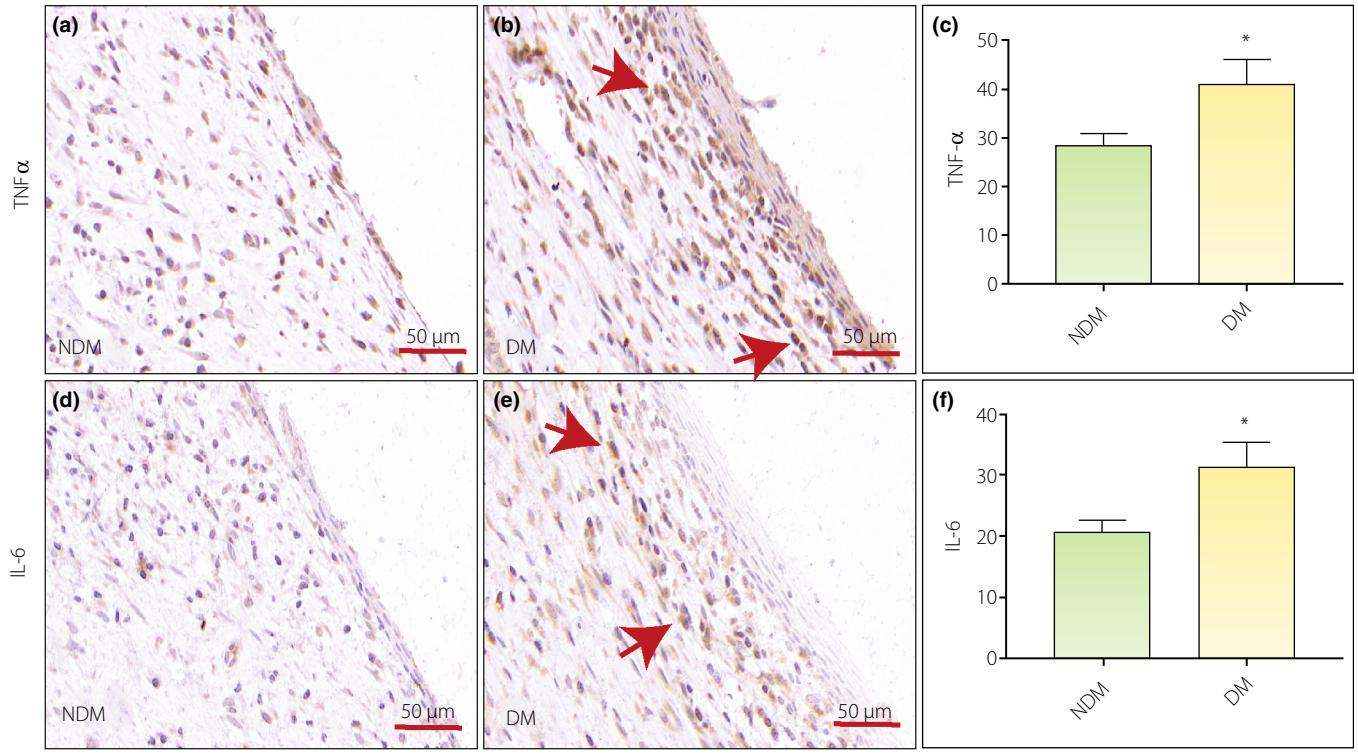

Figure 4 | Immunohistochemical staining of the hypomethylated genes, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in both the diabetes (DM) and non-diabetes (NDM) groups. TNF-α and IL-6 were positively expressed and significant increased under the sulcular/junctional epithelium area in the diabetes group compared with that in the non-diabetes group (red arrow shows positive expression).

significant increase in blood glucose was maintained throughout the observation period. Histological analysis showed that pancreas and renal tissues presented classic histopathological changes, such as atrophic islets of Langerhans, degranulated pancreatic β-cells and vacuolization of the renal tubular epithelial cells. The gingival (epithelial and connective) tissues in the lamina propria were also significantly affected by the diabetes mellitus induction, indicating that the periodontal tissue is a sensitive target for diabetes-induced changes. The present results are in agreement with previous studies in different diabetes mellitus animal models[31–33].

The acanthosis of the gingival epithelium, and the decreased and sparse rete pegs observed on diabetes mellitus induction in the present study could possibly be due to an increase in mitotic activity in the epithelial basal layer, promoting the differentiation of epithelial cells and flattening of rete pegs[34–36]. Several studies have shown that the onset of diabetes can lead to reduced gingival collagen synthesis, intracellular degradation and increased solubility[37–39], which could explain the irregular connective tissue changes observed in the diabetes mellitus group. In addition, we found that the number of blood vessels in the gingival tissue of the diabetes mellitus group significantly decreased when compared with those in the non-diabetes mellitus group, indicating that hyperglycemia affects angiogenesis, which is also consistent with previous reports[40,41].

As diabetes mellitus is closely associated with epigenetic changes[42], we investigated whether diabetes mellitus-dependent epigenetic changes affect the periodontal tissue, and hence might be a potential mechanism for the increased susceptibility to periodontitis. To the best of our knowledge, this is the first study addressing the effect of diabetes on periodontal tissue from an epigenetic perspective. We showed that diabetes mellitus significantly changed the DNA methylation level of the periodontal tissue, with 599 genes upregulated and 564 downregulated. Functional analysis of the hypermethylated genes revealed that the changes were closely related to lipid transport, hormone secretion, immune system, inflammatory response, angiogenesis and metabolic activity, which are vital processes in the development of periodontitis[27,43,44]. DNA methylation caused by diabetes mellitus could modify or disrupt the balanced status generally maintained in the ecological interactions between the host and the microbes, thus influencing the host susceptibility to the pathogenic bacteria. For example, the complement system, which can trigger, amplify, and regulate immune and inflammatory processes, is known to be deeply involved in the periodontal disease process[45]. Both GO and KEGG analysis showed that the complement system was affected by diabetes mellitus-dependent DNA methylation. The downregulation of *C1QS*, *MASP1*, *C6* and *C8* suggested the inhibition of the classical and lectin pathways of the

complement cascade, whereas the hypomethylation of *C3* and *C5AR1* showed that the alternative pathway of the complement system was still in an active state (Figure 5). *Porphyromonas gingivalis*, which is a key bacterial stimulus for periodontitis, can cause dysbiosis in the local periodontal environment with the help of complement C5AR1[46]. *P. gingivalis* can target C5AR and TLR2 subversive cross-talk, and induce a dysbiotic microbiota community. Once *C3* is also activated, periodontitis might develop[45]. Importantly, the alternative pathway plays a key role in chronic periodontitis[47,48]. Based on the present results, we can conclude that the periodontal tissue in the diabetes mellitus group reflects a compromised state that might be susceptible to the pathogenic mechanisms of *P. gingivalis* and other microorganisms. Consequently, periodontitis is likely to develop, and might explain the reason that diabetes mellitus patients have a higher prevalence of periodontitis. DNA methylation changes in the complement system reflect just one of the aspects affected in the diabetes mellitus group.

The alterations observed in inflammation-regulating cytokines also indicate that the gingival tissue in the diabetes mellitus group might be in a compromised state that is more susceptible to periodontal disease. Genes associated with inflammatory responses, such as *TNFA*, *IL-6* and *CCL22*, were hypomethylated. TNF-α and IL-6 are pro-inflammatory cytokines that can facilitate and amplify the inflammatory response through different pathways, and finally induce periodontal tissue degradation and bone resorption[49]. Overexpression of TNF-α and IL-6 has been regarded as a major contributor to periodontal disease[49–51]. In the present study, we observed high protein expression of the hypomethylated *TNFA* and *IL-6* genes under the junctional epithelium area in the diabetes mellitus group compared with those of the non-diabetes mellitus group. CCL22 is a known chemoattractant derived from dendritic cells and macrophages belonging to the CC chemokine family[52]. CC chemokines are crucial for the chemotaxis of monocytes/macrophages and lymphocytes, and regulate the switching of macrophages from M1 to M2 in the inflammation process, which play important roles in the inflammatory–immune response in periodontal disease[52]. Furthermore, CCL22-derived microparticles can recruit functional regulatory T cells to the periodontium and alleviate the inflammatory responses[53]. The high expression of CCL22, TNF-α and IL-6 all indicated that the periodontal tissue in the diabetes mellitus group was in a balanced, but more susceptible to periodontal disease status.

Overall, the present results show that diabetes mellitus might cause morphological and epigenetic changes in periodontal tissue, altering the defense status of the tissue and increasing its susceptibility to periodontal disease.

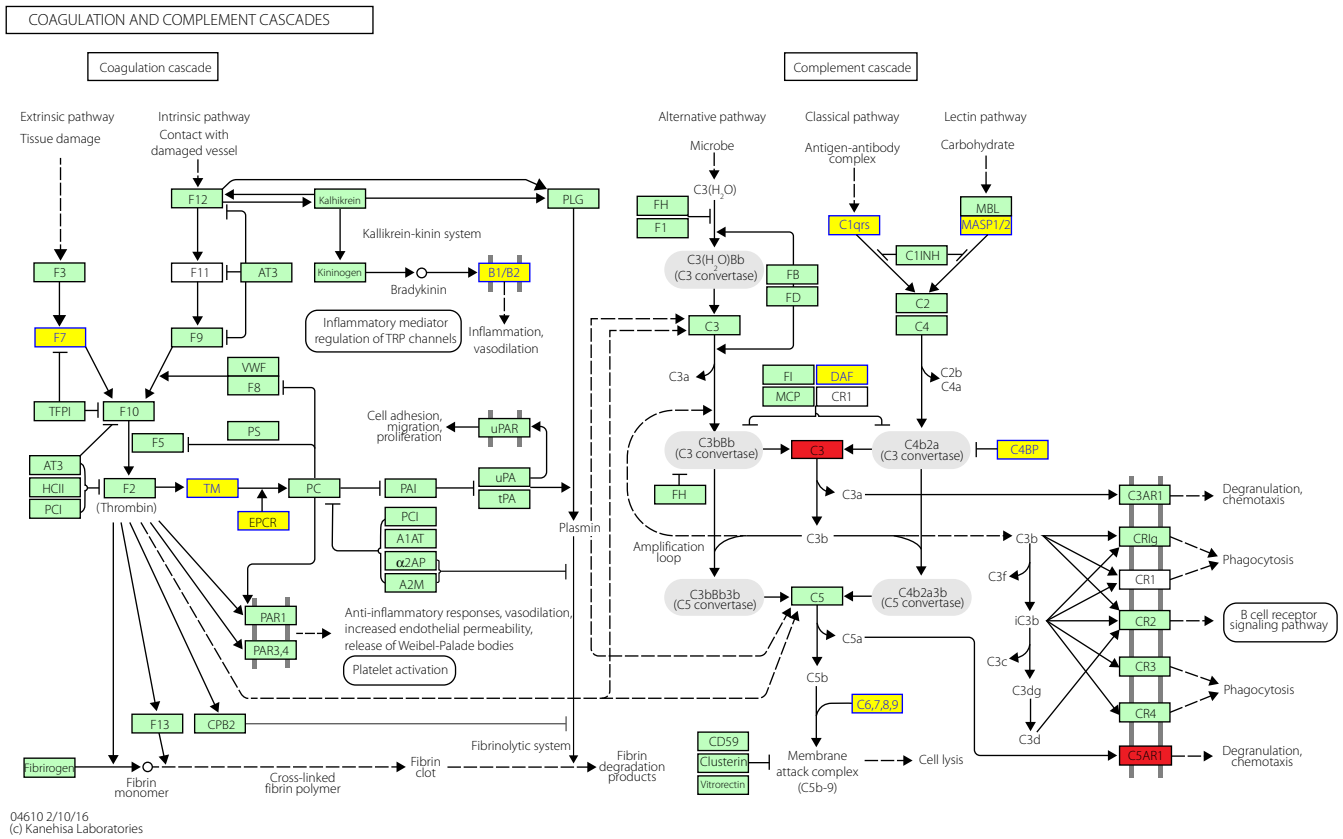

Figure 5 | Coagulation and complement cascades. Yellow color and red color represent hypermethylated and hypomethylated genes in the diabetes group, respectively.

It is worth mentioning that this was a pilot study based on a small sample size. More work needs to be carried out to further confirm the relationship between the diabetes mellitus and epigenetic changes of gingival tissue. Considering the minipig diabetes mellitus model is created by the injection of STZ, whether the epigenetic changes are induced by the drug itself or the diabetes mellitus model also deserves a further exploration.

STZ injection-induced diabetes mellitus causes significant morphological and DNA methylation changes in gingival tissue. DNA methylation of periodontal tissue could be one of the mechanisms for the increased susceptibility to periodontal disease in patients with diabetes.

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DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 | Histology changes of renal tubules in diabetes mellitus and non-diabetes mellitus groups (hematoxylin–eosin staining).
Table S1 | Top 20 categories of enrichment differentially expressed hypermethylated genes.
Table S2 | Top 20 categories of enrichment differentially expressed hypomethylated genes.
Table S3 | Interesting enrichment differentially expressed genes in both hyper- and hypomethylated biological processes.
Table S4 | Interesting Kyoto Encyclopedia of Genes and Genomes signal pathways of hypermethylated differently expressed genes.
Table S5 | Interesting Kyoto Encyclopedia of Genes and Genomes signal pathways of hypomethylated differently expressed genes.